The University of Alberta

Endonucleolytic Cleavage of Wheat Embryo Ribonucleates By Snake Venom Enzymes

by

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FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Endonucleolytic Cleavage of Wheat Embryo Ribonucleates by Snake Venom Enzymes," submitted by Barry Dean McLennan in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

ABSTRACT

At the inception of this investigation, there was little information available concerning limited endonucleolytic cleavage of naturally occurring RNA specimens, and it was of interest to investigate this problem with a view to defining controlled conditions under which RNA chains might be cleaved at specific phosphodiester bonds. This problem has been examined in the present thesis using wheat embryo rRNA and sRNA as substrates for (i) a venom endonuclease similar in its action to the classical pancreas RNase, and (ii) the venom exonuclease which had previously been noted to have an endonucleolytic action on low molecular weight substrates.

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Table of Contents

LIST OF TABLES

List of Illustrations

ABBREVIATIONS

PDase	snake venom phosphodiesterase (E.C. 3.1.4.1.)
PMase	an enzyme capable of hydrolyzing a monoester phosphate
rRNA	18S + 28S ribosomal ribonucleates
sRNA	ribonucleates soluble in molar sodium chloride
N	nucleoside [e.g. adenosine(A), guanosine(G), cytidine(C), or uridine(U)].
pN	nucleoside 5'-monophosphate
Np	nucleoside 2'(3')-monophosphate
N>p	nucleoside 2',3'-cyclic phosphate
pNp	nucleoside 3',5'-bis-phosphate
pN>p	nucleoside 2',3'-cyclic 5'-bis-phosphate
DEAE-cellulose	diethylaminoethyl-cellulose
A_{260}	absorbance at 260 millimicrons
s.u.	spectrophotometric units (absorbance x volume)
TRIS	tris(hydroxymethyl)aminomethane
DIOL	2-amino-2-methyl-1,3-propanediol
TCA	trichloroacetic acid
U-TCA	TCA containing uranyl acetate
'cyclic-ended' oligonucleotide	an oligonucleotide bearing a 2',3'-cyclic phosphate terminus
'open-ended' oligonucleotide	an oligonucleotide bearing a 3'-phospho-monoester terminus
Ψ	pseudouridine(5'-ribosyluracil)

PREFACE

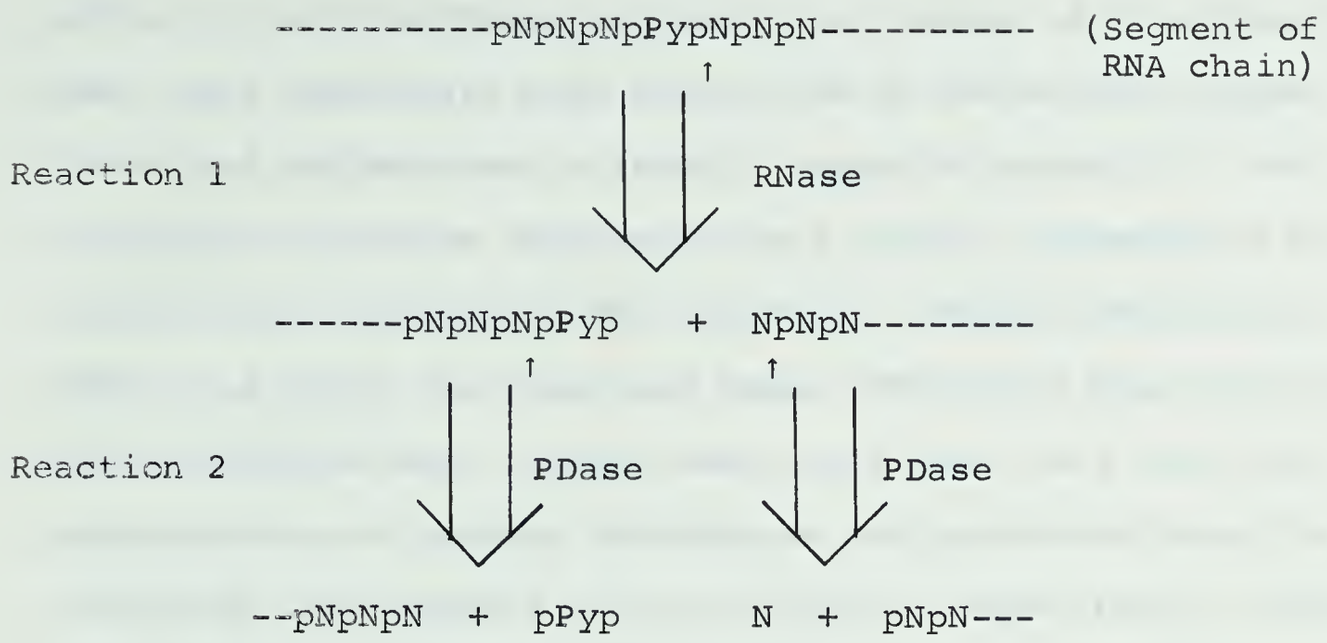
Nucleolytic enzymes from snake venom can be valuable agents with which to probe the structure of nucleates, but the utility of the enzymes for such studies is frequently limited by insufficient information about the details of their mode of action on naturally occurring polynucleotides. It is in this context that the studies of the thesis were designed.

The first comprehensive examination of the phosphatase activities in snake venoms was reported by Gulland and Jackson (1938), who concluded that most venoms contain both phosphomonoesterase (PMase) and phosphodiesterase (PDase) activities. In 1951, Hurst and Butler obtained the PDase activity free of PMase activity, and it is of historical interest that the venom 5'-nucleotidase could completely dephosphorylate the four deoxyribonucleotides present in PDase digests of deoxyribonucleates (DNA). This clearly indicated that the deoxyribonucleotides formed by the venom PDase were 5'-phosphates. Similarly, Cohn and Volkin (1952) showed that the venom PDase also liberated 5'-nucleotides by hydrolysis of ribonucleates (RNA). Thus, the venom enzymes were instrumental in showing that phosphodiester bonds in nucleates involved the 5'-carbon of deoxyribose in DNA and the 5'-carbon of ribose in RNA.

Helleiner and Butler (1955) characterized the venom PDase action on DNA as being primarily exonucleolytic. In subsequent investigations, Razzell and Khorana (1959), using low molecular weight model substrates, clearly elucidated the

mode of action of the exonuclease. However, the exonucleolytic action of snake venom PDase is not absolute since Felix et al. (1960) and Razzell and Khorana (1959) have reported that oligonucleotides having 3'-phosphomonoester termini, and cyclic oligonucleotides, respectively, are slowly hydrolyzed by the combined endonucleolytic and exonucleolytic action of PDase. There have been no recorded attempts to examine this endonucleolytic action of snake venom PDase using 18S + 28S RNA as substrate, and the studies that have been reported using sRNA as substrate, have frequently lacked the definition required to justify the conclusions which have been drawn (Nihei and Cantoni, 1963). It was thought, therefore, that it might be profitable to explore the endonucleolytic action of PDase on sRNA and 18S + 28S RNA from wheat embryo, and these studies are described in Part IV of the thesis.

The discovery of large amounts of nucleoside diphosphates in whole snake venom digests of RNA led Volkin and Cohn (1953) to postulate a branched-chain structure for RNA. However, Crestfield and Allen (1956) suggested that the nucleoside diphosphates did not arise from branch points in RNA but resulted from the action of venom PDase on the ends of chains created by a ribonuclease (RNase), which they postulated was present in whole venom. They also suggested that the venom RNase was similar to the classical RNase in its action on ribonucleates. Crestfield and Allen's interpretation of the origin of nucleoside diphosphates in whole snake venom digests of RNA can be schematically outlined as follows:



The reactions were visualized as occurring simultaneously in the whole venom digests. This interpretation suggested a rational basis for the measurement of chain ends produced by RNase action on RNA. Thus, if the RNase and PDase could be allowed to act in succession rather than in concert, the RNA could be subjected to controlled hydrolysis by the RNase (Reaction 1), and subsequently, after removal of the RNase, the fragments produced by the RNase could be digested with purified PDase devoid of the RNase activity in whole venom (Reaction 2). The nucleoside diphosphates and nucleosides would be formed in equimolar amounts from the chain ends of the fragments produced by the RNase, and would provide both a qualitative and quantitative measure of the cleavages which had been induced by the RNase.

In the course of attempts to develop such a method for measuring the chain ends produced by thermal fragmentation of RNA, further evidence for the existence of a venom RNase was obtained by Lane et al. (1963) who found that some

partially purified PDase preparations, devoid of 5'-nucleotidase, gave spuriously high quantities of nucleoside diphosphates and nucleosides in roughly equimolar quantity. The principal nucleoside diphosphate was uridine diphosphate and the principal nucleoside was adenosine. Witzel and Barnard (1962) had shown that pancreas RNase hydrolyzes PypA dinucleoside phosphates more rapidly than any others, and thus the preponderance of uridine diphosphate and adenosine among the nucleoside diphosphates and nucleosides, respectively, constituted further evidence for the existence of a venom RNase similar to pancreas RNase.

It seemed that the foregoing isolated observations might be usefully explored further in order to remove the existence of the venom RNase from the realm of deduction, and to compare it with the pancreas RNase in its action on RNA. Thus, the work described in Part I of the thesis deals with a study designed to develop a reliable method for qualitative and quantitative assessment of the chain ends produced by RNase action on RNA, and the studies of Part II describe some experiments designed to show that although the venom RNase activity can be obtained free of other activities, there is little prospect of economically purifying the enzyme in appreciable quantity because of the small amount present in venom. It is equally apparent, however, that in spite of the small amount present in venom, the venom RNase is just as potent as venom PDase in contributing to the nucleolytic action of snake venom on RNA, as judged by the conversion of RNA to acid-soluble split products.

At the inception of this investigation there was little available information about the relative rates at which the different susceptible bonds in naturally occurring RNA were hydrolyzed by RNases and therefore it was of interest to investigate this problem with a view to defining controlled conditions under which RNA chains might be cleaved at specific phosphodiester bonds to obtain fragments amenable to sequence analysis. The end-group analytical method developed in Part I seemed ideally suited to these purposes. In conjunction with oligonucleotide analysis of partial and complete digests, the end group technique has been utilized to examine the relative degree to which different susceptible bonds in wheat embryo rRNA and sRNA are broken when the RNA is subjected to various extents of hydrolysis by the venom or pancreas nucleases. The latter studies constitute the major part of the thesis investigation and are described in Part III.

Part I. End Group Analysis of Fragments Produced by Endonucleolytic Cleavage of Ribonucleates.

(i) Introduction.

Methods for the analysis of N- and C-terminal amino acid residues in native proteins are operationally the same as methods of analysis for endopeptidase cleavage since each scission of a peptide bond creates one new N-terminal and one new C-terminal amino acid residue. By contrast, the methods of analysis for the chain ends of native ribonucleates are operationally different from those needed to analyze for most types of endonucleolytic cleavage because the newly created chain ends are not the same type as found in native polymers.

The native chains of sRNA and rRNA preparations have a formal structure based on a repeating 5'-mononucleotide unit, $(pN)_n$ (Diemer, McLennan and Lane, 1966), whereas the products of most types of endonucleolytic fragmentation of native chains have a formal structure which can be considered to be composed of a repeating 3'-mononucleotide unit, $(Np)_n$. This can be schematically illustrated by the following sequence of reactions showing the endonucleolytic cleavage of a native RNA chain:

Scheme 1.

$(pN)_n$ = pNpN---pNpN---pNpN---pNpN---pNpN where n = 80(sRNA) and n = 1300(rRNA)

↑ ↑ ↑

m endonucleolytic scissions

1 pNpN---pNp

(m-1) N-----pNp = (m-1)$(Np)_n$

1 N---pNpN

The principal hydrolysis product is the $(Np)_n$ fragment since there are only two fragments which can be derived from terminal segments of a native chain while the number of $(Np)_n$ fragments increases with each endonucleolytic scission (i.e., for m = 10, there are 9 $(Np)_n$ fragments, and for m = 20, there are 19 $(Np)_n$ fragments). The $(Np)_n$ fragments most frequently arise from an intermediate structure bearing a terminal 2',3'-cyclic phosphate, which subsequently is opened to yield a terminal 2' or 3' phosphomonoester group (Fraenkel-Conrat and Singer, 1962):

$$(Np)_{n-1}N{>}p \xrightarrow{\text{Hydrolysis}} (Np)_{n-1}Np = (Np)_n$$

The nature of the chain ends of the fragments in Scheme 1 is determined by the mode of cleavage of the internucleoside bonds: between the 5'-carbon of ribose and the phosphorus atom. This mode of cleavage is characteristic of all types of chemical

hydrolysis by acids and bases, and of endonucleolytic cleavage by the largest class of endonucleolytic enzymes. There are, however, exceptions, in which the enzymic cleavage occurs between the 3'-carbon of ribose and the phosphorus atom to yield fragments whose formal structure is identical to that of the native chains:

Scheme 2.

$$(pN)_n = pNpN\text{---}pNpN\text{---}pNpN\text{---}pNpN$$

↑ ↑

m cleavages

$$(m+1)(pN)_n$$

Enzymes have been isolated (Heppel et al. (1957); Stevens and Hilmoe (1960); Yoneda (1964); and Linn and Lehman (1965)) which exhibit the type of attack shown in Scheme 2 but they are few in number compared with those which cleave RNA chains after the manner described in Scheme 1.

The analysis of chain ends in native ribonucleate chains is conveniently accomplished by hydrolysis of the RNA in alkali, which converts non-terminal residues to 2'- and 3'-nucleotides while terminal groups are released as nucleosides and nucleoside 2'(3'),5'-diphosphates.

$$(pN)_n = pNpNpNpN\text{------}pNpNpNpN$$

↑ ↑ ↑ ↑ ↑ ↑ ↑

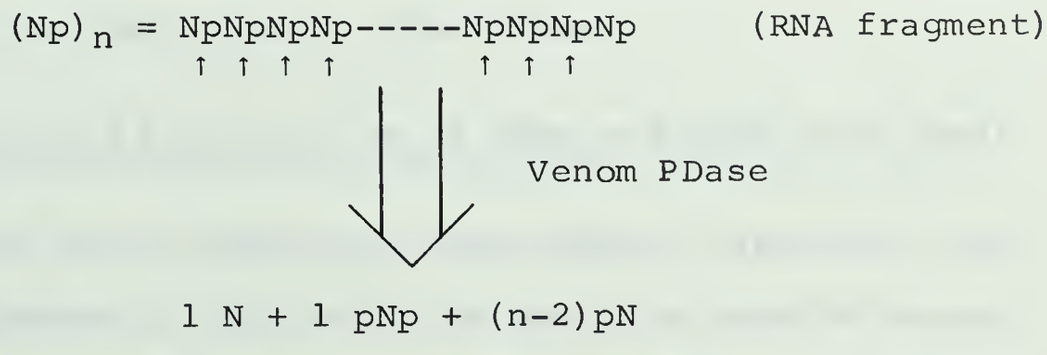
alkali hydrolysis

$$1\ N + 1\ pNp + (n-2)Np$$

This type of analysis has been widely used to show that the chain terminals in the sRNA from animal, plant and microbial sources are predominantly adenosine at the nonphosphorylated end and guanosine at the phosphorylated end. Just as N- and C-terminal analyses of native proteins have proved useful in determining the number of peptide chains per protein molecule, so, in the case of wheat embryo rRNA, the analysis for terminals by alkali hydrolysis of the RNA has suggested that the 28S component of rRNA may be composed of chains having the same length as the 18S component. The alkali hydrolysis procedure can also be used to measure the formation of end groups of RNA fragments created by endonucleolytic scission of native chains in the manner described in Scheme 2. This approach has been utilized in Part IV of this thesis.

An end group analytical method for determining the chain ends of fragments produced by the more common type of endonucleolytic cleavage shown in Scheme 1 has long been available in principle but has never been used in practice in studies of RNA hydrolysis. The principles of the method are outlined in Scheme 3:

Scheme 3.



and

$(Np)_{n-1}N>p$ = NpNpNpNp-----NpNpNpN>p (RNA fragment)

Venom PDase

1 N + 1 pN>p + (n-2)pN

The principles of Scheme 3 have been utilized in studying endonucleolytic scission of DNA chains (Koerner and Sinsheimer, 1957; Hurst and Becking, 1963; Lee and Zbarsky, 1966) and in the identification of oligonucleotides after removal of phosphomonoester groups but numerous difficulties are encountered in applying the method to studies of the endonucleolytic scission of RNA chains, and these obstacles are probably responsible for the scant use which has been made of this approach in studying ribonucleate hydrolysis.

The studies of this part of the thesis will deal with the development of a reliable procedure for the application of the principles of Scheme 3 to studies of ribonucleate hydrolysis and it will be apparent that several of the difficulties encountered are peculiar to the properties of the substrate, RNA, and to the potential interference by a specific ribonuclease which can contaminate venom PDase.

(ii) Isolation and Purification of rRNA and sRNA from Wheat Embryo.

Ninety gm of commercial wheat embryo (Thatcher variety) was suspended in 1200 ml of an emulsion made by mixing equal volumes of 0.05 M phosphate buffer (pH 6.8) and water-saturated phenol. After the suspension was shaken vigorously

for 15 minutes at room temperature and centrifuged at 1500 g for 20 minutes at 0°, it separated into an upper aqueous phase containing RNA and polysaccharides, a lower phenol phase containing protein and cellular debris, and an interphase of solid material. The upper aqueous phase was removed by suction, care being taken to avoid the interphase material.

Phenol was removed from the aqueous phase by ether extraction and traces of ether were subsequently removed by aeration of the solution at 4°. The solution was then made molar with respect to sodium chloride by the addition of solid salt and placed at 4° for 18 hrs. The sRNA and polysaccharides remained in solution while the rRNA precipitated.

The rRNA was collected by centrifugation at 0° and the resulting pellet was washed three times with 67% ethanol, three times with 95% ethanol and three times with ether, to remove salt, water, and ethanol, respectively. After the final ether wash, the powder was air-dried to constant weight at room temperature. The precipitation of rRNA from sodium chloride solution, washing with ethanol and ether, and air-drying were repeated in sequence twice more, and the resulting fine white powder was stored at -20°. The rRNA so obtained had an extinction coefficient (260 mμ) of 220-250 when dissolved in water to give a 1% solution and was therefore 95-100% pure. The yield of rRNA was about 1 gm from 90 gm of wheat embryo.

The sRNA was precipitated from the molar sodium chloride solution by the addition of two volumes of cold ethanol. The sRNA collected by centrifugation was freed of contaminating polysaccharides by methyl Cellosolve extraction according to

the method of Glitz and Dekker (1963). Further purification was achieved by precipitation with cetyltrimethylammonium bromide (Ralph and Bellamy, 1964) and by column chromatography on DEAE-cellulose (Glitz and Dekker, 1963). The product had an extinction coefficient of 200-220 at 260 mμ when dissolved to give a 1% aqueous solution. The yield of sRNA was about 150-200 mg from 90 gm of wheat embryo.

(iii) Isolation and Purification of PDase from Russell Viper Venom.

The PDase of Russell Viper Venom was purified free of other nucleolytic activities by the following procedure. An acetone fractionation similar to that used by Koerner and Sinsheimer (1957) was employed to remove effectively the bulk of the contaminating PMase, and final purification was accomplished by a modification of the cellulose column procedure described by Hurst and Butler (1951). Two hundred mg of venom purchased from Ross Allen's Reptile Institute (Florida) was dissolved in 12 ml of cold water and stirred for 2 minutes before the addition of 8 ml of 0.5 M ammonium acetate (pH 4.1). Acetone (14.5 ml) was added dropwise, with stirring, to the buffered venom solution, and the resulting suspension was stirred for 1 hour before collecting the precipitate by centrifugation at 4400 g for 15 minutes. Acetone (5.5 ml) was added dropwise, with stirring, to the clear supernatant solution obtained from the first centrifugation, and the stirring was continued for 15 minutes, before the precipitate was collected by centrifugation at 4400 g for 15 minutes. The supernatant solution from the second centrifugation was discarded and the pellets

obtained from two such preparations were combined and dissolved in 10 ml of water. The acetone fractionation was done at 4° but subsequent cellulose purification was performed at room temperature.

The PDase solution obtained from the acetone fractionation was assayed (p.14), diluted to 50 ml and passed through a 2.5 x 5 cm cellulose column prepared from 5 gm of analytical filter pulp (Carl Schleicher and Schuell Co.). The effluent was collected in 10 ml fractions and the column was washed with 10 ml aliquots of distilled water until the absorbance (280 mμ) had decreased to zero. The ultraviolet absorption spectrum of each of the fractions indicated the presence of acetone from previous purification steps and in order to remove this acetone the pooled fractions were evaporated in a flash evaporator at 20° until the volume was reduced to about 40 ml. When diluted to 60 ml this solution had an absorbance (280 mμ) of about 1. The solution was divided into ten ml aliquots and each aliquot of the enzyme solution was mixed with 10 ml of water before passage through a 2.5 x 2.5 cm cellulose column prepared from 1 gm of analytical filter pulp. The column was eluted with distilled water until the absorbance (280 mμ) of the effluents decreased to zero. Evaporation and fractionation of the pooled effluents on the small cellulose columns were continued under the conditions just described until the pooled enzyme solutions contained about 14 mg of protein (14 s.u. measured at 280 mμ). The resulting solution had suitable PDase activity and was devoid of other nucleolytic activities present in whole venom. Details relating

TABLE I

The Purity and Activity of Snake Venom PDase at Various Steps in the Purification Procedure

Purification Step	s.u. 280 mμ	Volume ml	Volume Assayed ml	Assay Value*	Total Activity	Specific Activity**
Whole venom	476	24	0.021	3.28	3749	8
After acetone fractionation	144	10	0.025	3.88	1552	11
After 5 gm cellulose column	60	70	0.1	2.27	1589	27
After 6 1-gm cellulose columns	36	131	-	-	-	-
After 4 1-gm cellulose columns	21	60	0.5	4.00	480	23
After 2 1-gm cellulose columns	13.5	54	0.5	3.14	339	25

*μmoles phosphodiester bonds cleaved ml^{-1} hr^{-1} in the standard assay (p.14).

**μmoles phosphodiester bonds cleaved ml^{-1} hr^{-1} mg^{-1} protein in the standard assay. (1 s.u. is presumed to represent 1 mg of protein.)

to a typical purification of PDase from 400 mg of venom are shown in Table I.

(iv) Hydrolysis of Native and Fragmented RNA Chains by PDase.

(1) Hydrolysis of native rRNA by PDase.

(a) Assay and conditions of PDase hydrolysis.

The activity of PDase preparations was measured by determining the rate of release of acid-soluble material during the hydrolysis of rRNA. The digestion mixture was prepared by mixing in rapid succession 1 ml of a 1% aqueous solution of RNA (ca. 22 μm of constituent nucleotide), 0.5 ml of 1 M ammonia - 1 M ammonium formate buffer (pH 9.2) and 0.5 ml of PDase solution. The resulting solution was incubated at 37° for one hour and the reaction was stopped by the addition of 4 ml of a 5% aqueous solution of TCA. The precipitate was removed by centrifugation and the absorbance of a 1:20 dilution of the supernatant solution was measured at 260 mμ. The total s.u. in the supernatant solution was calculated as follows:

volume of supernatant solution x dilution x absorbance (260 mμ)

or $5.8 \times 20 \times A_{260}$

A correction for the contribution of reagents to the absorbance of supernatant solutions amounted to 4 s.u. and was obtained by replacing the 0.5 ml of enzyme solution with 0.5 ml of water in the assay described above.

The activity of PDase solutions was expressed as hydrolysis rate in the following manner:

μmoles of phosphodiester bonds cleaved per hour per ml digest

$$= \frac{\text{s.u. in TCA supernatant solution} - 4}{11} \times \frac{1}{2}$$

where 11 is the mean mmolar extinction coefficient (260 mμ) of the four major nucleotide components of rRNA.

Alternatively, the rate of cleavage could be expressed as a percentage since the complete hydrolysis of 10 mg of rRNA gave 250 s.u. of TCA-soluble material. Thus,

$$\% \text{ hydrolysis} = \frac{\text{s.u. in TCA supernatant solution} - 4}{250} \times 100$$

The conditions for an end group analysis employing PDase were identical to those described above for the assay of the enzyme except that the scale of the digest was increased five-fold in order to obtain a measurable quantity of end groups. Thus, the digestion mixture for end group analysis was prepared by mixing 5 ml of a 1% aqueous solution of RNA, 2.5 ml of 1 M ammonia - 1 M ammonium formate buffer (pH 9.2) and 2.5 ml of PDase solution. The resulting solution was incubated at 37° for 24 hours.

An enzyme which hydrolyzed four μmoles of phosphodiester bonds per hour per ml of rRNA digest in the assay would thus hydrolyze 10 x 4 = 40 μmoles of phosphodiester bonds during the first hour of an end group analysis, and this activity was found suitable for ensuring complete digestion of the rRNA (110 μm of phosphodiester bonds) in end group analyses.

TABLE II

Eluents Used to Fractionate PDase Hydrolysates of rRNA on DEAE-cellulose

Compound	Net Charge at pH 7.8	Eluent
N	0	Water
pN	-2	0.085 M TRIS formate, pH 7.8, 7.3 M urea
pN>p	-3	0.35 M TRIS formate,* pH 7.8, 5.2 M urea
pNp	-4	
$(pN)_n$ where n>3	<-4	1 M pyridinium formate, pH 4.5

*Also elutes any unhydrolyzed dinucleotides (NpNp, pNpN) or trinucleotides (NpNpNp, pNpNpN) present in the hydrolysates.

(b) Analysis of digests.

The 24 hour hydrolysate was diluted to 100 ml with water and neutralized with concentrated formic acid before measuring the absorbance (260 mμ) of a 1:25 dilution of the solution. The neutralized hydrolysate was resolved into its components by fractional elution from an anion-exchange column.

The PDase hydrolysate of rRNA was applied to a 2.5 x 10 cm column of DEAE-cellulose(formate). The hydrolysis products were eluted from the column according to their net charge by increasing the concentration of the competing anion. The eluents used for the elution of the components found in PDase hydrolysates are shown in Table II. The inclusion of urea in the eluents facilitates the separation of these compounds on the basis of their net charge by reducing the non-ionic interaction between the nucleate derivatives and the DEAE-cellulose matrix (Tomlinson and Tener, 1963).

The components in the effluent fractions from DEAE-cellulose columns were desalted by adsorption to and elution from charcoal or DEAE-cellulose. The effluent containing nucleosides was adjusted to pH 5 by the addition of a few drops of formic acid and then an aliquot containing 0.1 to 1 μmole of nucleosides was passed through a 50 mg charcoal disc mounted between two layers of #545 celite in a glass column 2.5 cm in diameter. The column was washed with three 50 ml aliquots of water to remove residual salt, and the nucleosides were eluted from the charcoal with an eluent made by mixing 45 ml of water, 45 ml of 95% ethanol and 10 ml of pyridine.

The pyridine-ethanol effluent was evaporated in a flash evaporator at 40° and the resulting dry residue was recovered in a volume of 0.1 to 0.2 ml of water for paper chromatography.

The column fractions containing phosphorylated derivatives were diluted with water to reduce the salt concentration to about 0.03 M, and then desalted by passing the diluted solution through a 2.5 x 7.0 cm column of DEAE-cellulose (formate). The columns were carefully washed with about twenty 50 ml aliquots of water to remove residual salt and urea. The nucleotides were eluted from DEAE-cellulose by two 50 ml aliquots of 1 M pyridinium formate (pH 4.5), and the effluent was evaporated to dryness in a flash evaporator at 40°. The pyridinium salts of the nucleotides were converted to ammonium salts by evaporation in 0.6 M ammonium hydroxide solution. The recovery of pNp after desalting on DEAE-cellulose was about 90% while the recovery of N after desalting on charcoal was about 85%.

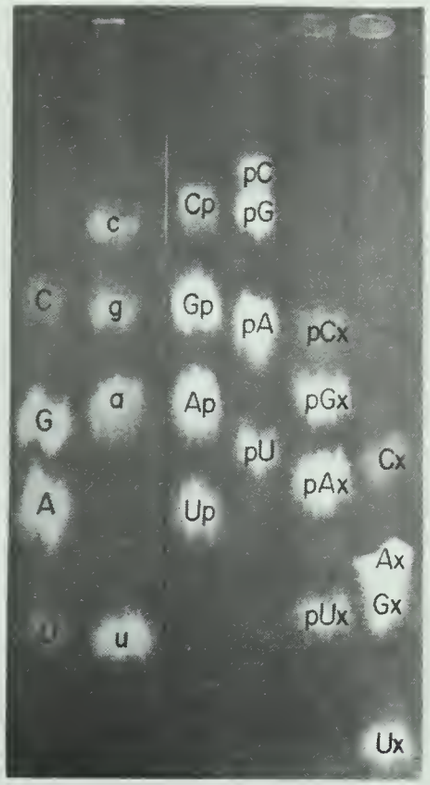
The individual components in each of the desalted fractions were resolved by one-dimensional paper chromatography (Lane, 1963). When separation was incomplete in one dimension, further separation was achieved by development in a second dimension with a solvent composed of 2 ml of isopropanol and 80 ml of saturated ammonium sulphate (cf. Markham and Smith, 1952). A permanent record of each chromatogram was obtained by making an ultraviolet contact photograph. The individual compounds were eluted from the paper with 3-8 ml of 0.1 M hydrochloric acid and characterized spectrally between 220 mμ and 340 mμ using a Bausch and Lomb Model 505 Spectrophotometer.

TABLE III

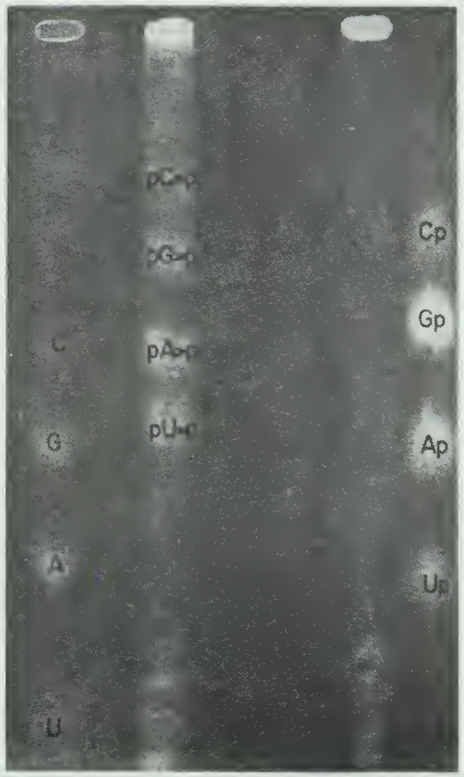
The Relative Amounts of Different Products
Formed by PDase Hydrolysis of Wheat Embryo rRNA

Compound	Constituent Nucleoside of Compound						
	A	G	C	U	Ψ	Nx	Total
	mole(s)/100 moles nucleotides						
N	0.056	0.050	0.027	0.028	-	-	0.16
pN	23.2	30.1	24.8	18.1	1.8	1.7	99.7
pN>p	0.028	0.023	0.050	0.052	-	-	0.15

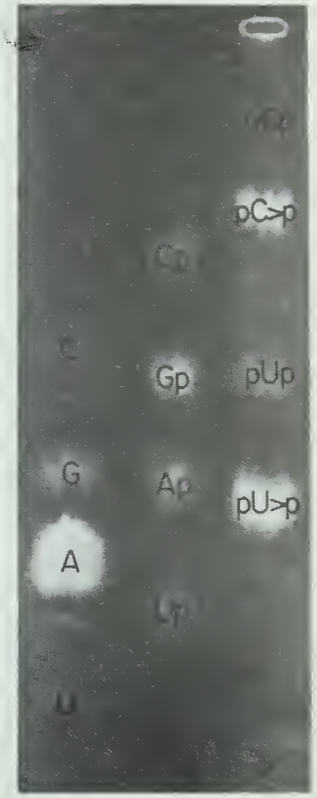
Figure 1.


pC
Cp
pG
c
C
g
Gp
pA
pCx
G
a
Ap
pGx
pU
Cx
A
pAx
Up
Ax
Gx
pUx
u
Ux

(a)


Cp
Gp
Ap
Up
C
G
A
U

(b)


pC>p
Gp
pUp
G
Ap
pU>p
A
U

(c)

Legend of Figure 1

Ultraviolet contact photographs of typical one-dimensional paper chromatograms. The origin of the chromatogram is, in each case, at the top of the photograph.

(a) A selection of useful separations which can be achieved by one-dimensional paper chromatography using ammonium sulphate-impregnated paper developed by a 76% ethanol-water solvent.

(b) The separation of nucleosides and nucleoside diphosphates present after PDase digestion of 50 mg of wheat embryo rRNA. The PDase preparation used for this digestion was devoid of 5'-nucleotidase and nuclease contaminants. The bulk nucleosides and bulk nucleoside diphosphates were separated from 5'-nucleotides by fractionation of the PDase digest on DEAE-cellulose, and the column fractions containing the nucleosides and nucleoside diphosphates were desalted prior to paper chromatography.

(c) The separation of nucleosides and nucleoside diphosphates present after PDase digestion of 5 mg of wheat embryo rRNA, which had been pretreated with venom ribonuclease (cf. Table XXII, 0.6 mg venom RNase per ml digest, 1 hr.).

The quantities of the individual compounds were corrected to quantitative recovery and expressed as mole(s) per 100 moles of total nucleotides in the PDase hydrolysates.

(c) Results of analyses.

These studies were performed in collaboration with Mr. Jans Diemer and the results of numerous analyses, which have been recorded elsewhere (Diemer, 1965), are summarized in Table III. The photograph of Figure 1a illustrates the paper chromatographic mobilities of many compounds pertinent to the studies in this thesis, and the photograph of Figure 1b shows the chromatographic resolution of the nucleosides and nucleoside diphosphates recovered from PDase digestion of 50 mg of rRNA.

The analysis for nucleotides was performed by direct two-dimensional paper chromatography of a PDase digest since the recovery of pG from anion-exchange columns is generally disproportionately lower than the recovery observed with other nucleotides. The quantities of the minor components (Lane, 1965) have been included in the analysis of the 5'-mononucleotides for purposes of statistical calculations entered in subsequent sections of the thesis.

(d) Interpretation of results.

These data have been described and discussed in the reprint appended to the thesis. The nucleosides and nucleoside diphosphates found in PDase digests of 18S + 28S RNA are thought to arise through a concerted sequence of reactions of the type shown on p. 3 (Preface), in which reaction 1 is

catalyzed by hydroxyl ion. These hydroxyl ion catalyzed cleavages are unavoidable at the pH value of 9.2 needed to achieve complete hydrolysis by PDase.

The nucleosides and nucleoside diphosphates formed by this spontaneous hydroxyl ion catalyzed hydrolysis of RNA during PDase hydrolysis must be subtracted from the values obtained in analyses for chain ends resulting from enzymic cleavage (Scheme 3, p. 9). However, the quantities of nucleosides, or nucleoside diphosphates, formed by spontaneous hydrolysis never exceed 0.2 mole % of the hydrolysis products and therefore the corrections are relatively small in terms of the 1 to 23 mole percent of nucleosides or nucleoside diphosphates which derive from enzymic cleavage in the different experiments of Part III of the thesis.

(2) Factors influencing the rate of hydrolysis and the quantity and quality of the products produced by the PDase digestion of RNA.

(a) Counterion of RNA.

The amount of nucleosides and nucleoside diphosphates arising from spontaneous hydrolysis of RNA during PDase hydrolysis is increased five-fold when the RNA is in the Mg^{++} rather than the Na^{+} counterion form. Although Mg^{++} is probably a cofactor for PDase action, exogenous Mg^{++} was omitted from digests in order to minimize the formation of nucleosides and nucleoside diphosphates during PDase hydrolysis.

(b) Type and concentration of buffer.

The rate of cleavage of RNA chains by PDase is dependent on both the ionic strength and type of buffer employed.

TABLE IV

Effect of Buffer Concentration on the Initial Rate of PDase Hydrolysis of rRNA

Buffer Concentration	Initial Rate of Hydrolysis* $\mu m\ hr^{-1}\ ml^{-1}$
0.25 M ammonia-0.25 M ammonium formate buffer (pH 9.2)	1.6
0.125 M ammonia-0.125 M ammonium formate buffer (pH 9.2)	2.7**

*Substrate concentration was 11 $\mu m\ ml^{-1}$ digest.

**This value is minimal since the reduced buffer concentration has the effect of decreasing the quantity of TCA-soluble material by about 10% in the PDase assay.

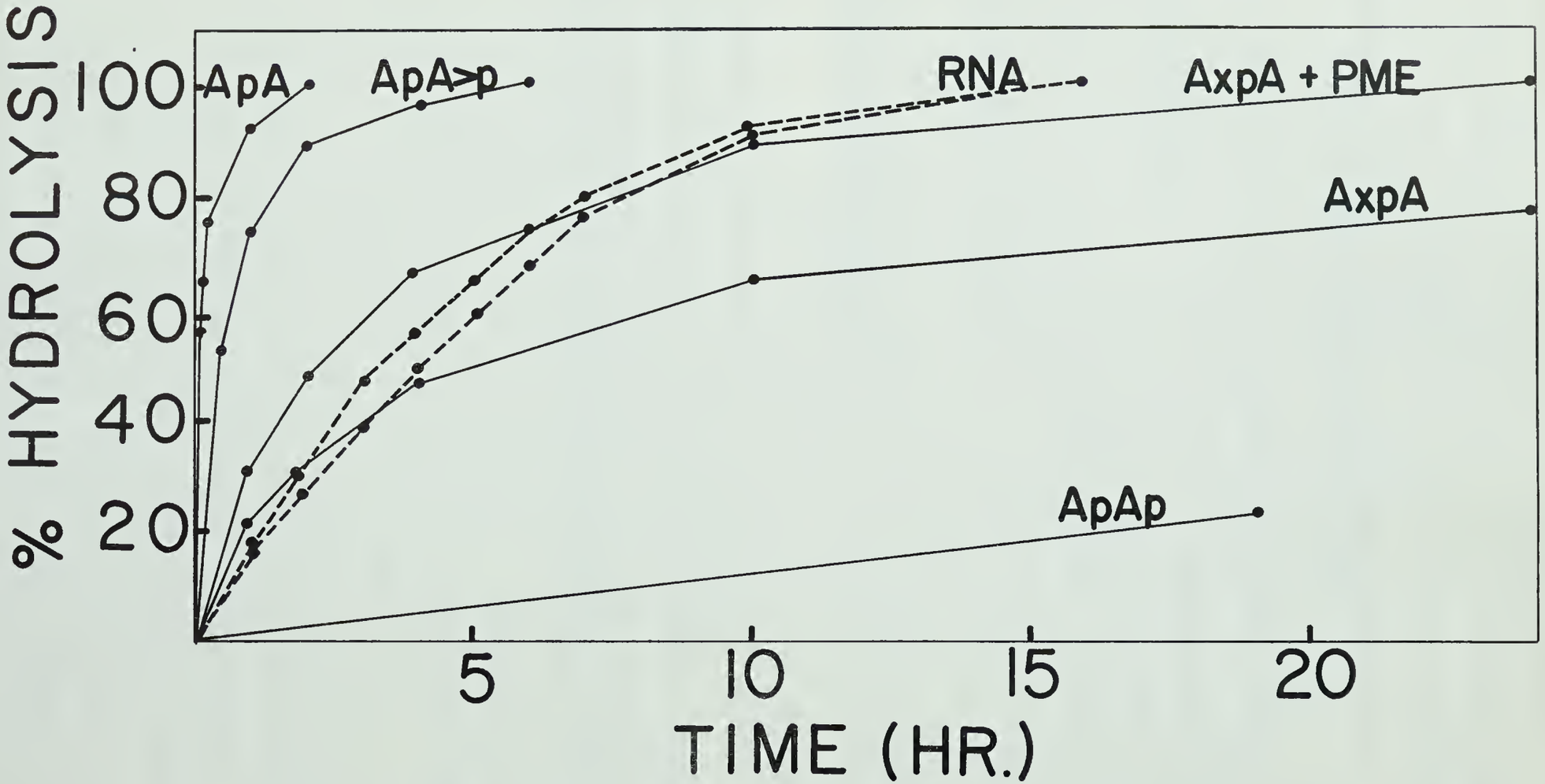
The employment of amino alcohol buffers such as TRIS or DIOL results in the formation of phosphodiesters in which one alcohol derives from the 5'-hydroxyl group of a nucleoside and the other derives from the buffer cation (Lane et al. 1963). These compounds can account for as much as 1 mole per cent of the hydrolysis products, and being neutral molecules at pH 7, they appear with nucleosides during anion-exchange fractionation of PDase hydrolysates. In order to avoid this side reaction, ammonia-ammonium formate buffers (pH 9.2) have been used for hydrolysis with PDase. The formate anion was used to avoid introducing anions such as chloride, which bind to DEAE-cellulose and ultimately interfere with desalting the nucleoside diphosphate fraction because of the formation of pyridinium chloride, a non-volatile salt.

The employment of suboptimal pH values for PDase digestion usually resulted in incomplete hydrolysis. Occasionally, even at pH 9.2, unhydrolyzed oligonucleotide residues, amounting to 0.2 to 1 mole percent of the hydrolysis products, were encountered. Analysis of these unhydrolyzed residues showed that they were rich in guanine and cytosine bases.

It was observed that either 0.125 M ammonia-0.125 M ammonium formate buffer (pH 9.2) or 0.25 M ammonia-0.25 M ammonium formate buffer (pH 9.2) provided adequate buffering capacity for PDase hydrolysis and both types of buffer were utilized in the present study. It is noteworthy that the initial rate of hydrolysis in the dilute buffer was substantially greater than that found with the concentrated buffer (Table IV), and this difference probably reflects the

Figure 2

The rates of hydrolysis of dinucleoside phosphates, dinucleotides and native ribonucleates by snake venom phosphodiesterase



The substrate concentration in terms of the concentration of constituent nucleoside, was 11 μm per ml of digest, buffered with 0.25 M ammonia-0.25 M ammonium formate (pH 9.2). The upper dashed curve depicts the hydrolysis of rRNA and the lower dashed curve depicts the hydrolysis of sRNA.

inhibition of PDase hydrolysis by a more compact secondary structure of the ribonucleate chains at high ionic strengths.

(c) Molecular size and type of substrate.

The rates of hydrolysis of the phosphodiester bonds in substrates of different molecular size are compared in Fig. 2. The time-courses pertinent to the present discussion are those for ApA (chain length = 2), sRNA (chain length = 80) and rRNA (chain length = 1300). Since the substrate concentration in terms of constituent nucleosides per ml of digest was the same in all cases, the initial effective substrate concentration (of chain ends) for the exonucleolytic action of PDase would be 2:80:1300 for ApA: sRNA: rRNA. As expected, ApA is hydrolyzed much more rapidly than sRNA and rRNA, but the hydrolysis rates of sRNA and rRNA are very similar. The fact that the hydrolysis rates of sRNA and rRNA are similar, in spite of a 20-fold difference in the initial concentration of chain ends, might be ascribed to inhibition by secondary structural organization in sRNA or to enhanced binding of rRNA by PDase. However, the studies of Part IV of the thesis indicate that endonucleolytic cleavages induced by PDase, cannot be overlooked in accounting for the comparable rates of hydrolysis of sRNA and rRNA.

(d) Integrity of polynucleotide chain.

The rate at which PDase hydrolyzes RNA depends on the integrity of the polynucleotide chains since rRNA which has been thermally degraded by storage at room temperature is hydrolyzed more rapidly than native rRNA. The effect of pre-induced chain scissions on the initial rate of hydrolysis is

TABLE V

The Effect of Chain Scissions on the Initial Rate of Hydrolysis of rRNA by PDase

rRNA	$S^{\circ}_{20,w}$	Initial Rate of Hydrolysis* $\mu m\ hr^{-1}\ ml^{-1}$
Freshly prepared	18 + 28	1.4
Stored -20°, 630 days	18 + 28	1.6
Stored +20°, 474 days	10	2.2

*Substrate concentration 11 $\mu m\ ml^{-1}$.

illustrated by the data of Table V. The increased hydrolysis rate of fragmented rRNA cannot be directly ascribed to an increased effective substrate concentration (of chain ends) since the effects of fragmentation on secondary structure, intermolecular interaction and enzyme-substrate binding cannot be assessed in relation to the rate of PDase hydrolysis. It is notable, however, that the bulk of the chain ends arising from thermal fragmentation have cyclic phosphate ends (Lane et al., 1963; Diemer, 1965) and such ends are good substrates for PDase action (cf. ApA>p in Fig. 2).

By contrast with the accelerated rate of PDase hydrolysis observed after limited fragmentation of rRNA, there is a severe inhibition of PDase hydrolysis after exhaustive fragmentation of RNA by pancreas RNase. The incomplete hydrolysis which is observed in such digests is partly due to the inhibition of PDase action by oligonucleotides bearing 3'-phosphomonoester groups (Razzell and Khorana, 1959a) and partly owing to the slow rate at which such oligonucleotides are themselves hydrolyzed by PDase. It can be seen from Fig. 2 that ApAp is very slowly hydrolyzed under the conditions which give complete hydrolysis of rRNA. It has been found in practice that the conditions used for complete PDase digestion of native rRNA always leave an unhydrolyzed residue when an exhaustive RNase digest is used as substrate for PDase hydrolysis. This difficulty can be overcome for purposes of end group analysis by increasing the PDase concentration in such digests and analyzing the residual oligonucleotides (mainly dinucleotides) either directly or by end group analysis after the removal of phosphomonoester termini (see Part III).

Figure 3

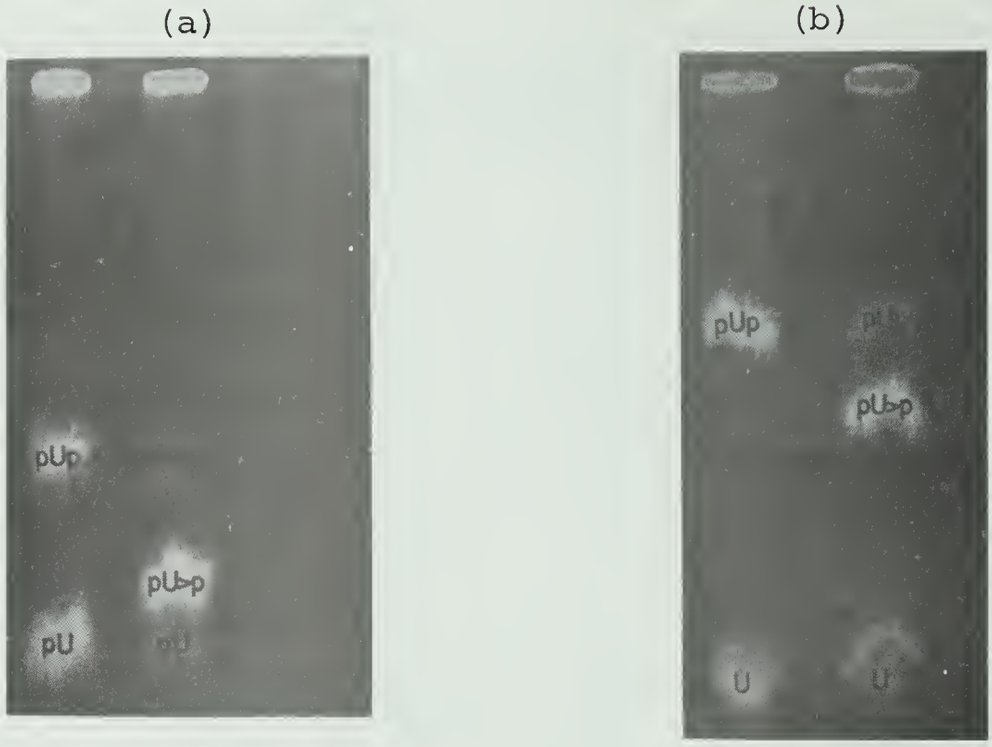
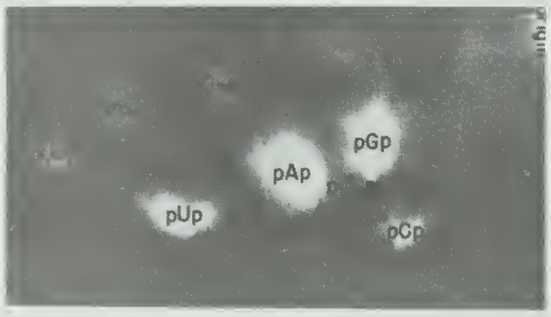


Figure 4



Legend of Figure 3

Ultraviolet contact photographs of one-dimensional paper chromatograms, which illustrate the destruction of pUp by purified PDase preparations, and by whole snake venom. The origin of the chromatograms is at the top of the photos.

(a) The resolution of the compounds present after treatment of pUp and pU>p with a PDase preparation devoid of 5'-nucleotidase and nuclease contaminants. The 2.5 ml digests, in 0.05 M ammonium formate buffer (pH 9.2), contained 0.2 μm substrate and 80 μg PDase per ml of digest, and were incubated at 37° for 24 hrs. prior to evaporation and redissolution in 0.1 ml of water for spotting on paper.

(b) The resolution of the compounds present after treating pUp and pU>p with whole snake venom. The 2.5 ml digests, in 0.05 M ammonium formate buffer (pH 9.2), contained 0.2 μm substrate and 360 μg venom per ml of digest, and were incubated at 37° for 24 hrs. prior to evaporation and redissolution in 0.1 ml of water for spotting on paper.

Legend of Figure 4

Ultraviolet contact photograph of a two-dimensional paper chromatogram, depicting the resolution of pNp and pN>p compounds. The origin is at the upper right-hand corner of the photo, and solvent development, in relation to the photo was right to left (first dimension) and top to bottom (second dimension).

Wheat embryo rRNA (50 mg) was mixed with about 1 μm of pNp compounds and then digested for 24 hrs. at 37° with PDase devoid of 5'-nucleotidase and nuclease contaminants. The pN>p and pNp compounds were separated from nucleosides and 5'-nucleotides by DEAE-cellulose column fractionation, desalted and spotted on paper.

(e) The destruction of pNp compounds by PDase.

It has been reported (Vanecko and Laskowski, 1962); Lane et al., 1963) that pNp compounds can be converted to pN compounds by purified preparations of snake venom PDase. When the 50 mg of RNA (110 μm of constituent nucleotides) is replaced by 1 μm of pNp compounds (0.25 μm of each of pAp, pGp, pCp and pUp) under the conditions used for end group analysis described in Part I (p.15), Diemer (1965) found that 50-70% of each of the nucleoside diphosphates was converted to the corresponding 5'-nucleotide with no evidence of nucleoside formation. The presence of 3 μm of pN compounds per ml of digest reduced the hydrolysis of each nucleoside diphosphate to less than 10%.

All PDase preparations used in the experiments reported in the thesis exhibited this same unusual property. The photographs in Fig. 3 illustrate a typical experiment showing the hydrolysis of pNp compounds by PDase and by whole venom (and, the stability of pN>p compounds under the same conditions).

Experiments were designed to assess the magnitude of the hydrolysis rate of pNp compounds relative to the rate of hydrolysis of bis-p-nitrophenyl phosphate by two purified PDase preparations and by whole snake venom. Since there was never any visible ultraviolet absorbing material in the nucleoside area of chromatograms after PDase treatment of pNp compounds, radioactive substrates were utilized to see if there might be detectable hydrolysis of Np and pN compounds of a lower order of magnitude than observed with pNp compounds.

TABLE VI

The Hydrolysis of Phosphomonoester Compounds by PDase Preparations

Substrate	Volume of Digest ml	Concentration**** of Substrate $\mu m\ ml^{-1}$	Control	Hydrolysis Rate ($m\mu m\ hr^{-1}$)			
				Venom $100\gamma ml^{-1}$	PDase 1 $12\gamma ml^{-1}$	PDase 2 $12\gamma ml^{-1}$	PDase 2 plus 10 μm pA ml^{-1}
RNA*	2	11	2.4	1400	370	370	-
bis**	1	1	0	114	95	123	-
pAp	0.5	0.25	0	0.58	0.48	0.50	-
	0.1	0.05 (1.5×10^4 cpm)	0	-	-	0.11	0.02
	0.1	0.005 (1.5×10^3 cpm)	0	-	-	0.02	0.0008
A2p	0.1	0.070 (2×10^6 cpm)	0.034	0.096	0.080	0.040	-
A3p	0.1	0.046 (2×10^6 cpm)	0.030	0.068	0.036	0.0012	-
pA	0.1	0.080 (2×10^6 cpm)	0.037	8.7×10^{3}***	0.054	0.0020	-

*rRNA. Assay described on p.14.

**bis-p-nitrophenyl phosphate. Assay described in Part II, p.31.

***Same as assay conditions for RNA except that RNA was replaced by an equal weight of pA and release of inorganic phosphate was measured after one hour at 37°.

****The concentration of all substrates, including the radioactive substrates, has been based on ultraviolet absorbance measurements of stock solutions.

All hydrolyses were performed in 0.25 M ammonia-0.25 M ammonium formate buffer, pH 9.2, and the hydrolyses of nucleotides and RNA were performed with 500 γ protein ml^{-1} of venom digests and 60 γ protein ml^{-1} of PDase digests. The nucleotide digests were sampled at 15 hours in order to obtain suitable degrees of hydrolysis. Hydrolysis of bis-p-nitrophenyl phosphate was conducted at 100 γ protein ml^{-1} of venom digest and 12 γ protein ml^{-1} of PDase digest in order to obtain an initial rate of hydrolysis at 1 hour. The values entered in the table for the hydrolysis rate of the nucleotides and RNA have been calculated from the measured values presuming the extent of hydrolysis is linearly dependent on time, and protein concentration. Diphosphate digests were analyzed by one-dimensional paper chromatography (Lane, 1963) and monophosphate digests were analyzed by paper electrophoresis in 1 M formic acid (after neutralization and addition of carrier nucleotides and nucleosides where necessary). Nonradioactive compounds were eluted and quantitated by absorbance measurements while radioactive compounds were excised and counted in scintillation vials. All experiments were performed in duplicate and sometimes in triplicate and the results of duplicate experiments were in excellent agreement. Radioactive monophosphates were obtained from Schwarz Bioresearch and were purified by electrophoresis in 1 M formic acid to remove endogenous nucleosides prior to use in these experiments. Radioactive pAp was prepared by subjecting uniformly labelled ^{14}C-yeast RNA (Schwarz Biochemicals) to spontaneous hydrolysis prior to PDase digestion. The PDase digest was then treated with alkali to convert ^{14}C-pN>p compounds to ^{14}C-pNp compounds and ^{14}C-pAp was recovered by column and paper chromatographic purification.

The results of these experiments (B. G. Lane, unpublished observations) are summarized in Table VI. The rate of hydrolysis of pAp is seen to be extremely slow even when it is compared with the hydrolysis rate of bis-p-nitrophenyl phosphate, which is known to be a poor substrate for PDase (Razzell and Khorana, 1959a). The slow hydrolysis rate for pAp is accentuated further when it is noted that the pH value of 9.2 used for these experiments is substantially removed from the pH optimum (pH 8.3) for the hydrolysis of bis-p-nitrophenyl phosphate by PDase (Gulland and Jackson, 1938). The rate of hydrolysis of pNp compounds relative to bis-p-nitrophenyl phosphate is, however, remarkably similar for the purified PDase preparations and whole venom, and this might suggest that the hydrolysis of the pNp compounds is effected by PDase itself. The limited purity of the PDase preparations does not, however, preclude the possibility that there might be some other effector of the hydrolysis. It seems very probable that the venom hydrolysis of pNp compounds reflects a secondary catalytic activity which is unrelated to the primary activity of the effector since the extremely slow rate of the hydrolysis of pNp compounds by venom could only be classified as negligible in any conceivable "in vivo" context. In this sense, then, the venom PDase is as suitable, and perhaps more suitable, than other venom constituents, as a candidate for the role of the effector of this unusual hydrolysis.

Sulkowski *et al*. (1963) alluding to a deficit of pNp compounds in PDase hydrolysates of oligonucleotides bearing 3'-phosphomonoester groups suggested that the destruction of

TABLE VII

The Stability of pNp Compounds in PDase Hydrolysates of rRNA

pNp	Amount of pNp in the Hydrolysate*	
	Before Digestion μm	After Digestion μm
pAp	0.31	0.32
pGp	0.27	0.24
pCp	0.09	0.10
pUp	0.16	0.19

*The conditions for the hydrolysis of rRNA were identical to those described earlier (p.14) except for the addition of the pNp compounds. The actual quantities of the pNp compounds recovered from the PDase hydrolysate are quoted.

pNp compounds was due to contamination by a nonspecific phosphatase present in whole venom. However, since the nonspecific phosphatase was shown to be at least as active in hydrolyzing Np and pN compounds as it was in hydrolyzing pNp compounds, the failure of PDase to hydrolyze pN compounds in the experiments reported by these authors would seem to indicate that there was no contamination by the nonspecific phosphatase in their highly purified preparations.

Whether or not the pNp ——> pN conversion is effected by PDase itself, it seemed important to establish that inhibition of the pNp hydrolysis by pN is adequate to ensure that the compounds are stable under the conditions used for PDase hydrolysis of RNA. For this purpose, a PDase digest of rRNA was supplemented with 0.1-0.3 μm of each of pAp, pGp, pCp and pUp at the beginning of the digestion, and after incubation for 24 hours at 37° the hydrolysate was fractionated in the usual way. The photograph of Fig. 4 (p.23) shows the two-dimensional separation of the recovered pNp compounds together with the pN>p compounds arising from spontaneous hydrolysis of the rRNA during PDase digestion. The results of the experiment are summarized in Table VII and it is apparent that there was no appreciable destruction of any of the compounds. The stability of the pNp compounds under these conditions is even more apparent when it is noted that the figures quoted are actual recoveries and have not been corrected for losses which can be incurred during the analytical procedure.

Under the conditions of the experiment just described, there was about 1 μm of pNp and about 110 μm of pN in the 10 ml

digest. This corresponds to the analysis of a fragmented RNA preparation in which the nucleoside diphosphates derived from chain ends constitute about 1 mole percent of the hydrolysis products. It can be seen from Table VI that the percent destruction (20-50%) of pAp in the absence of pN is not greatly affected by its concentration over the range 0.005-0.25 μm pAp per ml of PDase digest, and there is, in any event less than 5% destruction in the presence of 10 μm pN per ml of digest in all cases.

Thus, it is clear that destruction of pNp compounds is not significant in PDase hydrolysates over a range of total pNp varying from 0.05-2.5 μm per 10 ml of digest (i.e., about 0.05-2.5 mole percent of the hydrolysis products in end group analyses). These latter observations indicate that destruction of pNp by PDase would not likely be an important factor in the failure to detect pNp compounds in PDase hydrolysates of native RNA. The quantity of pNp compounds formed by the spontaneous hydrolysis (about 10%) of the pN>p compounds in such digests would only correspond to 0.1 x 0.12 = 0.012 mole percent of the hydrolysis products and would have escaped detection in any case since the analyses were performed on only 50 mg of rRNA.

(f) Contamination of snake venom PDase by PMase or RNase activities.

PMase and RNase are the enzymes most frequently found to contaminate PDase preparations from Russell Viper Venom. The presence of either of these enzymes in PDase preparations can be ascertained by an inspection of the products formed when RNA is hydrolyzed by the PDase preparation.

TABLE VIII

The Results of End Group Analyses When PDase Preparations Containing PMase or Nuclease Contaminants Are Used to Effect the Hydrolysis of RNA

PDase Contaminant	RNA Digestion Time	A	G	C	U	Total
		mole %				
PMase	24	1.02	1.14	0.25	0.53	2.94
	48*	2.38	2.88	0.51	1.16	6.93
Venom RNase	24	1.26	0.07	0.06	0.06	1.45
	48*	1.29	0.08	0.06	0.06	1.49

*Incubation was extended to 48 hours with the addition, at 24 hours, of a second 2.5 ml aliquot of PDase.

In the absence of PMase, the total mole percent of nucleosides should equal the total mole percent of nucleoside diphosphate (about 0.1 to 0.2 m % of the total hydrolysis products). If the quantity of nucleosides greatly exceeds the expected amount, while the quantity of nucleoside diphosphates is as expected, the presence of PMase in the PDase preparation is indicated and the PDase can be purified by further passage through cellulose as described earlier (see p. 13). The presence of PMase in PDase preparations can be confirmed, using the large-scale digest described on p. 15 by the addition of a further 2.5 ml of PDase to the 24 hour hydrolysate and extension of the digestion time to 48 hours. Subsequent analysis will reveal an increase in quantity of nucleosides over that obtained after 24 hours if PMase is present.

Contamination of PDase with venom RNase will lead to increased quantities of adenosine and uridine diphosphate in PDase hydrolysates of RNA. Further incubation after supplementing the digest with a second aliquot of enzyme will not alter the quantities of nucleosides and nucleoside diphosphates unless PMase is present in addition to the venom RNase. Table VIII shows typical nucleoside quantities obtained when PDase is contaminated with PMase and venom RNase.

(3) Routine procedure adopted for the end group analysis of fragmented RNA.

In view of the wide array of factors which can influence the nature and extent of the hydrolysis of RNA by PDase, it is useful to summarize the conditions and precautions

observed in the preparation of the digests and in the analysis of the hydrolysis products. It is important to isolate rRNA in the sodium counterion form, to store the ethanol-ether dried powder at -20° and to check the integrity of the substrate by sedimentation analysis. Undegraded rRNA should be used for enzyme activity assays since thermally degraded RNA gives a higher assay value than native rRNA. The use of ammonium formate buffer avoids side reactions encountered with aminoalcohol cations and interference by chloride anions in desalting procedures.

It is important to note that the products produced by limited fragmentation of rRNA are suitable for end group analysis by PDase hydrolysis, but that exhaustive fragmentation necessitates the removal of 3'-phosphomonoester end groups by PMase treatment of the hydrolysis products. Although PupPyp compounds are very resistant to PDase hydrolysis under the conditions used for end group analyses, they must nevertheless be accounted for in such analyses since up to 50% of the total PupPyp compounds can remain unhydrolyzed in PDase digests.

The most difficult aspect of the utilization of snake venom PDase for end group analysis of RNA fragments relates to the purity of the PDase, since the conclusions drawn from end group data can be very misleading if trace amounts of PMase or venom RNase contaminate PDase preparations.

Part II. The Existence of a Ribonuclease in Russell Viper Venom.

(i) Introduction.

Preliminary observations (Lane et al., 1963) suggested that an endonuclease existed in snake venom which displayed a preferential specificity for PypA phosphodiester linkages in ribonucleates (see Preface). This conclusion conformed with the suggestion by Crestfield and Allen (1956) that a nuclease similar to pancreas RNase existed in whole venom and received further support from the observations of Witzel and Barnard (1962) who had shown, in studies of the hydrolysis of dinucleoside phosphates, that pancreas RNase exhibits a preferential specificity for PypA linkages. The deductions about the possible existence of a venom RNase were given substance by the successful resolution of the ribonucleolytic activity of whole venom into discrete exo- and endonucleolytic fractions by gel-filtration on G-100 Sephadex.

If the venom RNase resembled pancreas RNase in its action on ribonucleates, it seemed possible that it might also show the characteristic acid-stability of the pancreas enzyme, and as a consequence, the venom RNase might be freed of other activities by direct acid treatment of whole venom. This acid treatment of whole venom proved to be a very effective means of recovering the venom RNase free of interfering activities (McLennan and Lane, 1965). A similar approach has recently

been utilized by Rushizky et al., (1964) in their studies of the RNases from various organisms.

Since the venom RNase usually contaminates PDase prepared by acetone fractionation of whole venom, it seemed possible that it might be enriched in one of the acetone fractions, and by exploring this possibility, it was found that the venom RNase could also be obtained free of other nucleolytic activities by the acetone precipitation technique.

Having demonstrated the independent existence of a venom RNase and having shown that its action was similar to that of the presumed contaminant RNase occasionally found in PDase preparations, it seemed worthwhile to utilize the demonstrated acid stability of the RNase to confirm that it could be recovered after acid inactivation of the PDase in those preparations presumed to have been contaminated by a venom RNase. The results of end group analysis of the RNA fragments produced by the acid-treated PDase preparation showed that the contaminant enzyme had, in fact, survived the inactivation of the PDase.

Some basic similarities between the venom and pancreas RNases are described in this section of the thesis but a more detailed comparison of their activities is presented in Part III.

(ii) Separation of the Endo- and Exonucleases of Snake Venom by Gel-Filtration on G-100 Sephadex.

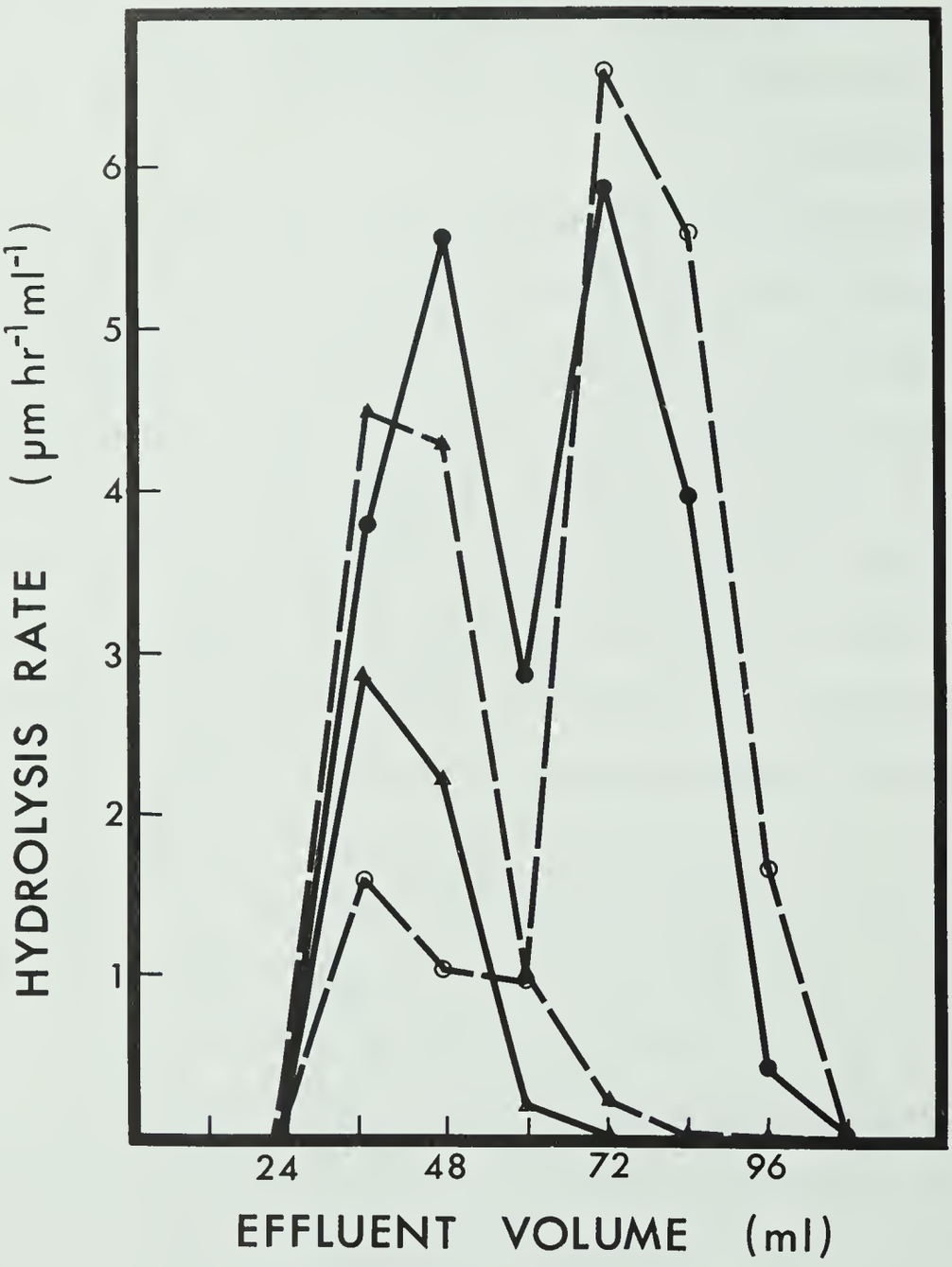
The exo- and endonucleolytic activities of Russell Viper Venom can be resolved by gel-filtration on G-100 Sephadex (Pharmacia [Canada] Ltd.). A 2 ml aliquot of a 2.75%

aqueous solution of whole venom was applied to a 2.5 x 17 cm column prepared by allowing a three-day water suspension of G-100 Sephadex to pack under gravity flow. Removal of protein from the column by water elution was continued until the absorbance (280 mμ) of the 12 ml effluent fractions had decreased to zero.

The method of Helleiner (1955) was utilized to distinguish between endo- and exonuclease activities. Each fraction from the column was assayed for its ability to convert RNA into fragments soluble in (i) TCA and (ii) TCA containing 0.25% uranyl acetate (U-TCA). Whereas large oligonucleotide fragments (up to octanucleotides or perhaps larger) are soluble in TCA, only mononucleotides and limited quantities of dinucleotides are soluble in U-TCA, and as a consequence, the products of endonuclease digestion are much more soluble in TCA than in U-TCA while the products of exonuclease digestion are nearly equally soluble in both TCA and U-TCA.

The conditions for assay of TCA-soluble fragments produced at pH 9.2 have been described and the conditions for assay of U-TCA-soluble products were identical with those used in the TCA assay except that precipitation of digests was effected with 5% TCA containing 0.25% uranyl acetate. As further evidence that the exonuclease activity was attributable to PDase, the fractions were assayed using bis-p-nitrophenyl phosphate as substrate. The assay for PDase activity was performed by measuring the increment in absorbance (400 mμ) when 1 μm of bis-p-nitrophenyl phosphate in 0.65 ml of water (adjusted to pH 8) was mixed with 0.25 ml of 1 M ammonia-

Figure 5



The resolution of snake venom exonucleoltyic and endonucleolytic activities by gel-filtration on G-100 Sephadex

- TCA assay (pH 9.2)
- bis-p-nitrophenyl phosphate assay
- TCA assay (pH 7.6)
- U-TCA assay (pH 9.2)

ammonium formate buffer, pH 9.2, and 0.1 ml of the effluent fraction, and incubated for one hour at 37°. The production of TCA-soluble fragments at pH 7.6 (as well as pH 9.2) was also measured in order to show that the venom endonuclease activity was, unlike PDase, almost equally active at pH 7.6 and pH 9.2 as would be expected for an enzyme resembling pancreas RNase.

The results of fractionation of whole venom on G-100 Sephadex and of the assays for the different activities are summarized in Fig. 5. It is apparent that the exo- and endonucleolytic activities were nearly equal when assessed in terms of the rate of formation of acid-soluble products from RNA. Since this work was done, a report has appeared in which the fractionation of venom endo- and exonucleases was achieved by passage of whole venom solutions through a sulphocellulose column (Babkina and Vasilenko, 1964).

(iii) Recovery of Snake Venom RNase Free of Other Nucleolytic Activities by Acid Treatment of Whole Venom.

Twenty-five to 1000 mg of Russell Viper Venom in 5 ml of water were mixed with 5 ml of 0.25 M sulfuric acid and slowly stirred for 45 minutes at room temperature before neutralization with 0.2 to 0.3 ml of 10 M potassium hydroxide. The precipitate which frequently formed was removed by centrifugation at 2,000 rpm in a clinical centrifuge for 2 minutes, and the supernatant solution was stored at 4°. The resulting solutions had suitable venom RNase activity for the hydrolysis of ribonucleates and their RNase activity was stable for several months when stored at 4°. The venom RNase activity

was measured in a manner similar to that described for the assay of PDase except that the 1 M ammonium formate buffer (pH 9.2) was replaced with 1 M TRIS formate (pH 7.6) (see p. 14).

The combined TCA and U-TCA assays at pH 9.2 showed that the solution of acid-treated venom was predominantly endonucleolytic in its action on RNA and was devoid of PDase and PMase activities as judged by its action on RNA and 5'-mononucleotides, respectively.

(iv) Recovery of Snake Venom RNase Free of Other Nucleolytic Activities by Acetone Precipitation of Whole Venom.

The venom RNase activity could be freed of all other nucleolytic activities by an acetone fractionation procedure similar to that described for the isolation and purification of snake venom PDase (p. 12). Acetone (7.0 ml) was added dropwise, with stirring, to the clear supernatant solution obtained from the first centrifugation and the stirring was continued for 15 minutes at 4° before the precipitate was collected by centrifugation at 4400 g for 15 minutes. Then, 18.5 ml of acetone was added dropwise with stirring to the clear supernatant solution from the second centrifugation, and stirring was continued for 15 minutes before collecting the pellet by centrifugation at 4400 g for 15 minutes. The pellet was dissolved in 10 ml of water and the resulting solution was shown by TCA and U-TCA assays to have a predominantly endonucleolytic activity and it was devoid of PDase and PMase activity as judged by its action on RNA and 5'-nucleotides, respectively.

The yield of enzyme activity was only one-eighth as great as that obtained by acid treatment of whole venom and this indicates that the nuclease is either partially inactivated or fractionally distributed during acetone precipitation of whole venom. It is noteworthy that most of the nucleolytic activity found in the 67% acetone pellet (Cohn, Volkin and Khym, 1957) is probably ascribable to the venom RNase.

(v) Recovery of Snake Venom RNase Free of Other Nucleolytic Activities by Acid Treatment of Partially Purified PDase Preparations.

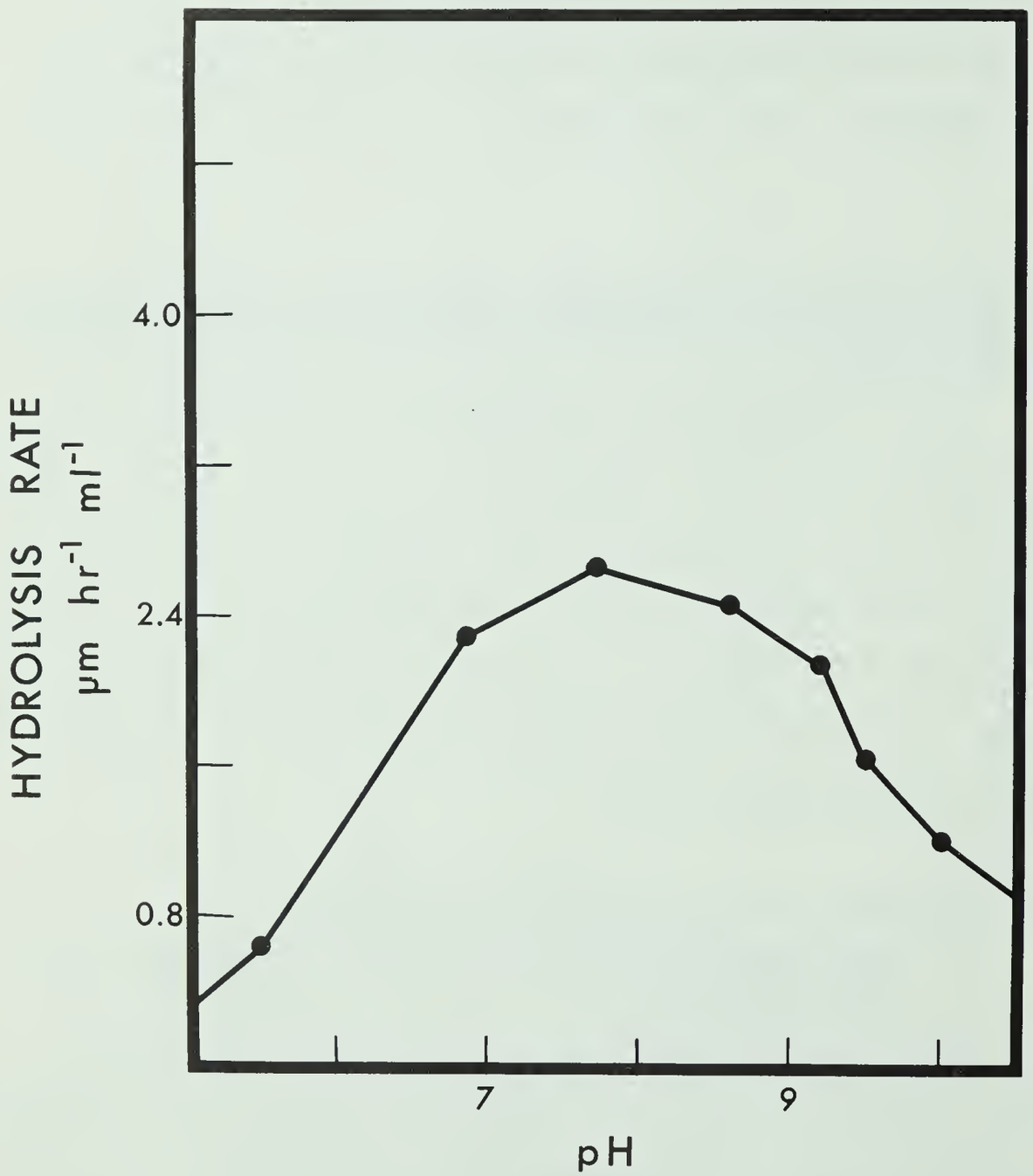
A 5 ml aliquot of a partially purified PDase preparation presumed to contain venom RNase, was mixed with an equal volume of 0.25 M sulfuric acid and stirred slowly for 20 minutes at room temperature before neutralization with 0.3 ml of 10 M potassium hydroxide. The acid treatment inactivated the PDase activity and the remaining enzyme activity was shown to be venom ribonuclease (p.35).

(vi) Some Properties of the Snake Venom RNase.

(1) Acid stability and the effect of temperature on acid stability.

Assay for the production of TCA and U-TCA-soluble products showed that the venom RNase activity in acid-treated venom solutions was undiminished during incubation in 0.125 M sulfuric acid at room temperature between 5 and 45 minutes and the activity was the same whether acid treatment was performed at 0° or room temperature. If the activity obtained after acid treatment of whole venom is taken to reflect the

Figure 6.



The pH dependence of Venom RNase activity in the TCA assay.

full venom RNase activity, then it is possible to assess how much of the venom RNase is present per gram of venom. The acid-treated venom solution was compared with a measured weight of pancreas RNase in terms of the ability to produce TCA-soluble products from RNA at pH 7, and it was found that there is probably not more than 25 μg of venom RNase per gram of whole venom if the venom RNase has the same molecular weight and turnover number as pancreas RNase.

(2) pH dependence.

The effect of hydrogen-ion concentration on the activity of the venom RNase is shown in Fig. 6. There is a broad optimal pH range from pH 7.0 to pH 8.5 and the continuity of the line representing the enzyme activity suggests that there were no specific effects by the different buffers used (acetate, phosphate, TRIS, ammonia or ethanolamine buffers).

(3) Specificity.

The mode of cleavage of ribonucleates by the venom RNase prepared by any of the methods just described appears to be identical, and it is concluded that all three methods of isolating the venom ribonuclease yield essentially the same enzyme activity. A schematic representation of the manner in which the venom RNase, as well as many other endonucleases, hydrolyze RNA is shown in Part I (p. 7). A comparison of the nucleoside and nucleoside diphosphate compounds derived from the end groups of RNA fragments created by the action of venom RNase prepared by acid treatment or acetone fractionation of whole venom, or by acid treatment of partially

TABLE IX

The Nucleosides and Nucleoside Diphosphates
Derived from the Chain Ends of RNA Fragments Created by
the Endonucleolytic Action of Venom Ribonuclease.

Source of the Endonuclease	A	G	C	U	Total N	pA>p	pG>p	pC>p	pU>p	Total pN>p
	mole %									
Acid-treated venom	1.51	0.06	0.10	0.05	1.7	-	-	0.62	1.15	1.8
67% acetone precipitate of whole venom	1.07	0.09	0.06	0.05	1.3	-	-	0.46	0.84	1.3
Acid-treated partially purified PDase	1.79	0.07	0.07	0.03	2.0	-	-	0.65	1.40	2.0

The general procedure for estimating N and pN>p compounds is described in Part III. In the above experiments, the substrate (wheat embryo rRNA) was treated for one hour. The photograph of Figure 1c (p.18) shows the chromatographic resolution of the nucleosides and nucleoside diphosphates recovered from the chain ends of RNA fragments created by the endonucleolytic action of venom RNase.

purified PDase preparations, is shown in Table IX. The preponderance of A among the nucleosides and of pU>p among the nucleoside diphosphates suggests that the RNA fragmentation is being catalyzed by the same enzyme in all three cases.

The venom RNase is a true endonuclease since it produces about three times more TCA-soluble than U-TCA-soluble material from RNA after one hour, and fractionation of the hydrolysis products on DEAE-cellulose showed that there were no detectable mononucleotides in the digests of Table IX, even when digestion was extended to 24 hours.

The venom endonuclease is a ribonuclease since there was no detectable hydrolysis of DNA or heat-denatured DNA when DNA or heat-denatured DNA replaced RNA in standard assays.

(4) Prospects for purification.

The venom RNase activity parallels the pancreas enzyme in its acid stability, pH dependence and preferential specificity for PypA linkages in RNA. Since the quantity of RNase in venom is small, it was felt that there would be little purpose in purifying it unless it could first be demonstrated that the venom RNase possessed properties which would distinguish it from the pancreas RNase, which is commercially available as a crystalline enzyme in almost unlimited quantity and at reasonable cost. The studies of Part III show that the venom RNase is an interesting model for comparison with the pancreas RNase, and therefore its purification is perhaps justified, in spite of the costly

investment in the starting material (snake venom). Acetone precipitation, acid treatment of whole venom and gel-filtration on G-100 Sephadex have been suggested as potentially useful purification steps in this section of the thesis.

Part III. The Cleavage of Wheat Embryo rRNA and sRNA by Snake Venom and Pancreas RNases.

(i) Introduction.

There is a widespread search among plant, animal, and microbial sources for endonucleases having a restricted specificity for the cleavage of phosphodiester bonds in RNA (Rushizky et al., 1964; Glitz and Dekker, 1964; and Linn and Lehman, 1965). In only a few cases (Rushizky et al., 1962) however, are details available regarding the preferential specificity which known endonucleases might exhibit toward a limited fraction of the susceptible bonds in RNA. The most striking example of preferential specificity during limited digestion is the cleavage of alanyl and tyrosyl sRNA molecules into two nearly equal halves by T_1-RNase (Zamir et al., 1965; Madison et al., 1966).

While the action of pancreas RNase on RNA has been well-defined in terms of the nature of the products produced by exhaustive hydrolysis, there have been few attempts (Rushizky et al., 1961; Litt and Ingram, 1964) to examine the products of partial hydrolysis.

The studies in this part of the thesis deal with a comparison of the products resulting from limited scission of RNA by the pancreas and venom RNases. The comparison is based upon (i) quantitative and qualitative measurements of phosphodiester bond scission by end group analysis of the hydrolysis products, and (ii) qualitative and quantitative assessment of

the mononucleotides and oligonucleotides found among the hydrolysis products.

Obstacles encountered in the application of end group methods have been described and discussed in Part I of the thesis. Fractionation of the oligonucleotides present in RNase digests of RNA also presents a number of problems which have been overcome largely through the efforts of Tomlinson and Tener (1963) and Rushizky and Sober (1964). Rushizky et al. (1964a) and Bartos et al. (1963) have described methods for fractionating mixed oligonucleotides on DEAE-Sephadex and DEAE-cellulose, respectively. The procedures are basically extensions of the original finding by Tomlinson and Tener (1963) that the inclusion of urea in eluents results in an elution of oligonucleotides from DEAE-cellulose which is primarily dependent upon the net charge of the compounds bound to the anion exchanger. Oligonucleotides are eluted from DEAE-Sephadex solely on the basis of their net charge at pH 7.6 while elution from DEAE-cellulose depends primarily on the net charge and secondarily on the purine/pyrimidine ratio of the oligonucleotides (Bartos et al., 1963; Tomlinson and Tener, 1963). Since exhaustive RNase hydrolysis of RNA yields oligonucleotides of the general structure $(Pup)_{n-1}$ Pyp (see Part I, p. 7), either of the above methods could be employed to fractionate such hydrolysates.

However, partial RNase hydrolysis of RNA produces oligomers with a variable purine/pyrimidine ratio and consequently, only DEAE-Sephadex can be used to fractionate such hydrolysates on the basis of net charge. Thus, since the

studies described in this part of the thesis involved the analysis of both exhaustive and partial RNase hydrolysates, the method described by Rushizky *et al.* was adopted.

Although the DEAE-Sephadex method separates oligonucleotides according to their net charge, the components having the same net charge are not homogeneous with respect to chain length because partial hydrolysates contain both "open" and "cyclic" ended oligonucleotides. Thus, even though NpNp and NpNpN>p compounds differ in chain length, they are found to be eluted in the same fraction because they have the same charge at pH 7.6 (i.e., -3).

(ii) Analytical Methods.

(1) Assay and conditions used for hydrolysis of RNA by the venom and pancreas RNases.

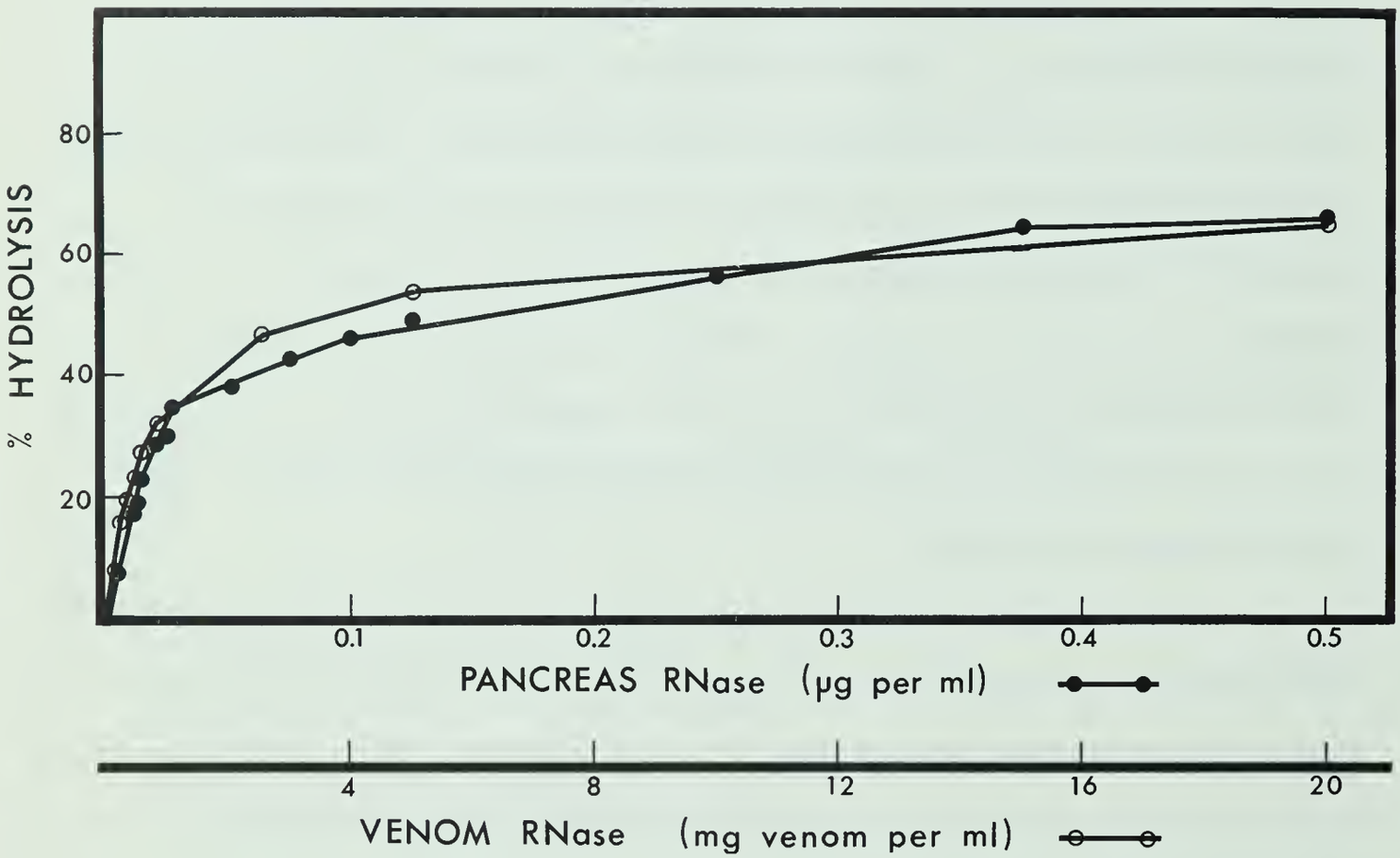
The activities of the venom and pancreas RNase solutions employed to effect the fragmentation of rRNA and sRNA were measured by an assay identical to the one described for assaying snake venom PDase activity (Part I, p. 14), except that the 1 M ammonium formate buffer (pH 9.2) was replaced by 1 M TRIS formate (pH 7.6). The conditions used for partial digestion of RNA by the RNases were the same as those used in the assay, except that the scale of the digest was increased five-fold in order to obtain readily measurable amounts of end groups. Thus, 50 mg of rRNA or sRNA in 5 ml of water was mixed with 2.5 ml of 1 M TRIS formate (pH 7.6) and 2.5 ml of RNase solution. All incubations were conducted at 37° for one or for 24 hours and the nuclease action was stopped by shaking the digest for 2 minutes with an equal volume of water-saturated

phenol (Rushizky et al., 1964a). After centrifugation of the aqueous suspension for 5 minutes at 2,000 rpm in a clinical centrifuge, the emulsion separated into an upper aqueous layer, a lower phenol layer and an interphase of solid material. The aqueous layer containing the RNA hydrolysis products was removed by suction, reextracted with phenol and recentrifuged. The resulting aqueous layer was removed, freed of traces of phenol by ether extraction, and then desalted on a 2.5 x 10 cm column of DEAE-cellulose(formate). The RNA hydrolysis products were eluted by 6 x 50 ml of 1 M pyridinium formate, and the effluent was evaporated to dryness in a flash evaporator at 40°. The dry residue was dissolved in 25 ml of 0.6 M ammonium hydroxide and after evaporation, the dry residue was dissolved in 5 ml of water. The recovery of hydrolysis products was 90-100%.

For complete digestion of RNA with pancreas RNase, the hydrolysates contained 50 γ RNase per ml of digest and digestion was permitted to proceed for 24 hours at 37°. Subsequent phenol treatment of the digest was omitted prior to PDase digestion.

The pancreas RNase used in these experiments was purchased from Nutritional Biochemicals Co. The working solutions were freshly prepared from a stock RNase solution (1 mg RNase per 1 ml water) and assayed just before use. It was found that serial dilutions of the stock solution of RNase lost activity when stored at 4° or at -20° whereas the stock solution retained full activity when stored at -20°. A venom RNase solution (100 mg Russell Viper Venom/ml) was prepared

Figure 7



The enzyme-concentration dependence of the activity of venom and pancreas RNases in the TCA assay

as described in Part II (p. 32) and then dialyzed against cold running tap water for two hours. The dialyzed venom RNase preparation was stored at 4° without loss of activity. The activity of this stock solution was equivalent to that found for acid-treated venom at a concentration of 80 mg per ml.* As may be seen from Fig. 7, the enzyme-concentration dependence of activity in the TCA assay was similar for the pancreas and venom RNases over a 100-fold range in concentration.

(2) End group analysis of the products formed by RNase digestion of RNA.

The hydrolysis products produced by limited RNase digestion of 50 mg of RNA were dissolved in 5 ml of water and mixed with 2.5 ml 1 M ammonium formate buffer (pH 9.2) and 2.5 ml of purified snake venom PDase devoid of contaminant PMase or nuclease activities. The digestion with PDase was allowed to proceed for 24 hours at 37° before analysis of the digest for nucleosides and nucleoside diphosphates as described in Part I (iv). In terms of the economy of PDase, it should be noted that for the highest RNase concentrations used to effect partial hydrolysis in these experiments, the digests could be scaled down as much as ten-fold because of the nearly twenty-fold increment in end groups over the amount formed at

---

*It was found that sulphate in the venom RNase solutions interfered with the resolution of oligonucleotides on DEAE-Sephadex and therefore it was removed by dialysis. The removal of sulphate did not significantly alter the activity of the venom RNase but the dilution resulting from dialysis reduced the enzyme concentration by about 20%. Prolonged dialysis of solutions containing venom RNase (and pancreas RNase (Taborsky, 1959) must be avoided since the RNase slowly diffuses through Visking cellophane tubing.

the lowest RNase concentrations. However, in order to easily detect small amounts of 3'-linked pyrimidine nucleoside end groups, the digests were never scaled down more than five-fold.

In experiments in which complete hydrolysis of RNA had been effected by pancreas RNase, the concentration of PDase used for end group analysis was increased six-fold over that used for partial hydrolysates, and, for the reasons just mentioned, the hydrolysis products from 5 mg of RNA sufficed for the analysis. The unhydrolyzed oligonucleotide residue in such PDase digests (Part I, p. 22) consisted primarily of dinucleotides and was eluted from DEAE-cellulose together with pNp compounds. The contribution of the oligonucleotides to the total end group analysis was assessed by direct paper chromatographic resolution of the four dinucleotides and two nucleoside diphosphates. Alternatively, the oligonucleotides were eluted together with pNp compounds by 1 M pyridinium formate (pH 4.5) and the mixture of pNp compounds and oligonucleotides was treated with PMase before digestion with PDase. The purine nucleosides produced in this way provided a measurement of the contribution of the oligonucleotides to the total end group measurement.

(3) The oligonucleotide analysis of the products of RNase digestion.

(a) Preparation of DEAE-Sephadex columns.

After removing the fines, a 24 hour water-suspension of 100 gm of DEAE-Sephadex (A-25 medium, Pharmacia [Canada] Ltd.) was filtered on a Buchner funnel and the resulting

"cake" was suspended in 1 liter of 0.5 N hydrochloric acid. This mixture was filtered, the resulting "cake" was washed in 1 to 2 liters of water and then the "cake" was suspended in 1 liter of 0.5 N sodium hydroxide. After removal of the sodium hydroxide by filtration, the DEAE-Sephadex was washed with 1 to 2 liters of water and then neutralized by suspension in 1 liter of 0.5 N TRIS chloride buffer (pH 7.6). The DEAE-Sephadex was then washed thoroughly with 0.02 M TRIS chloride (pH 7.6)--7.6 M urea--0.14 M sodium chloride ("starting" buffer).

A slurry of DEAE-Sephadex (chloride) in 0.02 M TRIS chloride buffer (pH 7.6) was packed by gravity flow to a height of 35 cm in a glass column 2.5 cm in diameter. The column was equilibrated by passage of 1 liter of the "starting" buffer.

(b) Resolution of RNase hydrolysates on DEAE-Sephadex and desalting of the effluent fractions.

Products produced by partial hydrolysis of RNA (700 s.u.) were applied to an equilibrated DEAE-Sephadex column in a volume of 2-5 ml of water and washed into the column with 2 x 3 ml aliquots of "starting" buffer. The nucleate derivatives were eluted from the column by a linear gradient from 0.14 to 0.28 M sodium chloride. The limiting and starting solutions contained 0.02 M TRIS chloride buffer (pH 7.6). Two liters of starting buffer and 2 liters of limiting buffer were placed in each of two cylinders connected by a section of polythene tubing. Effluent samples containing 40 ml were collected by means of a fraction collector (Microchemical

Figure 8

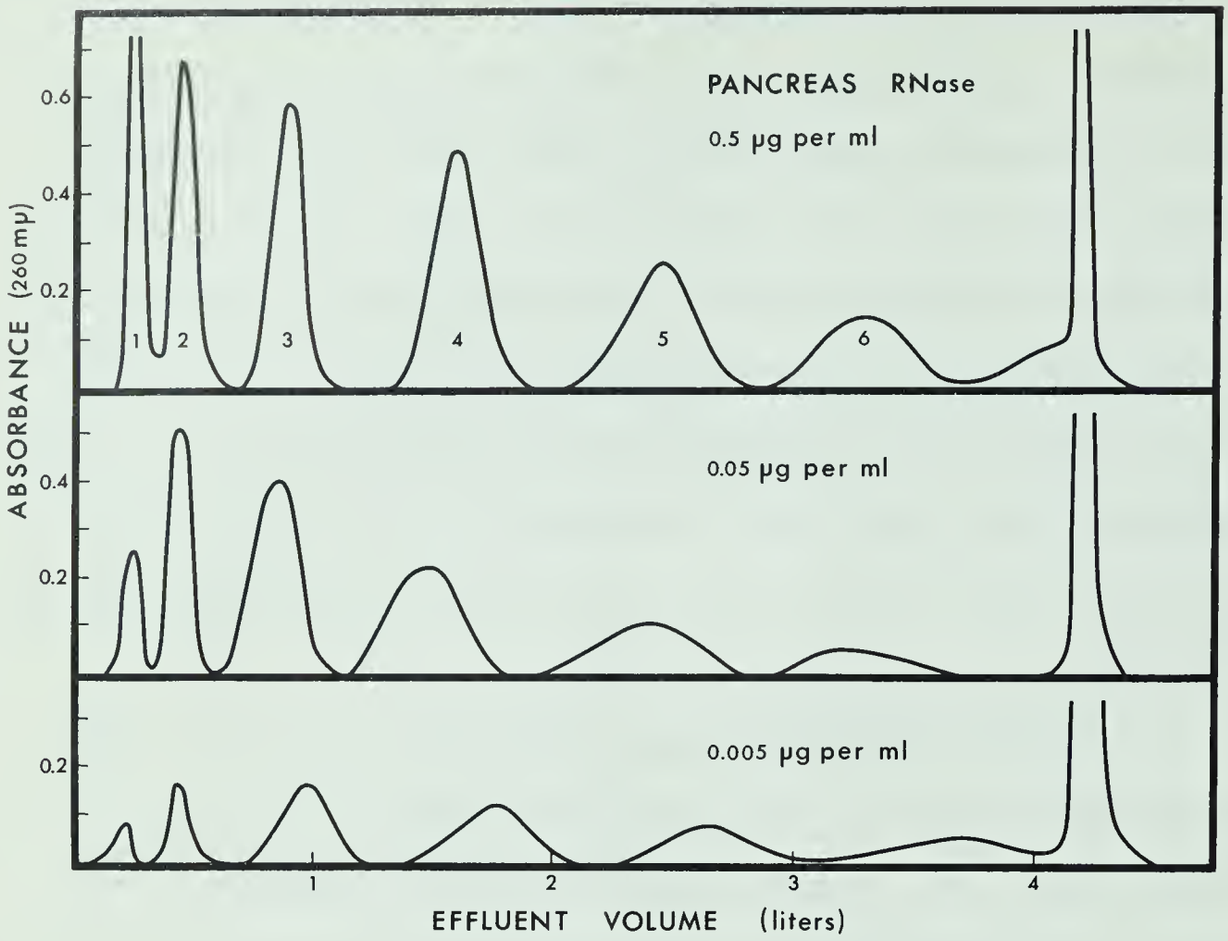
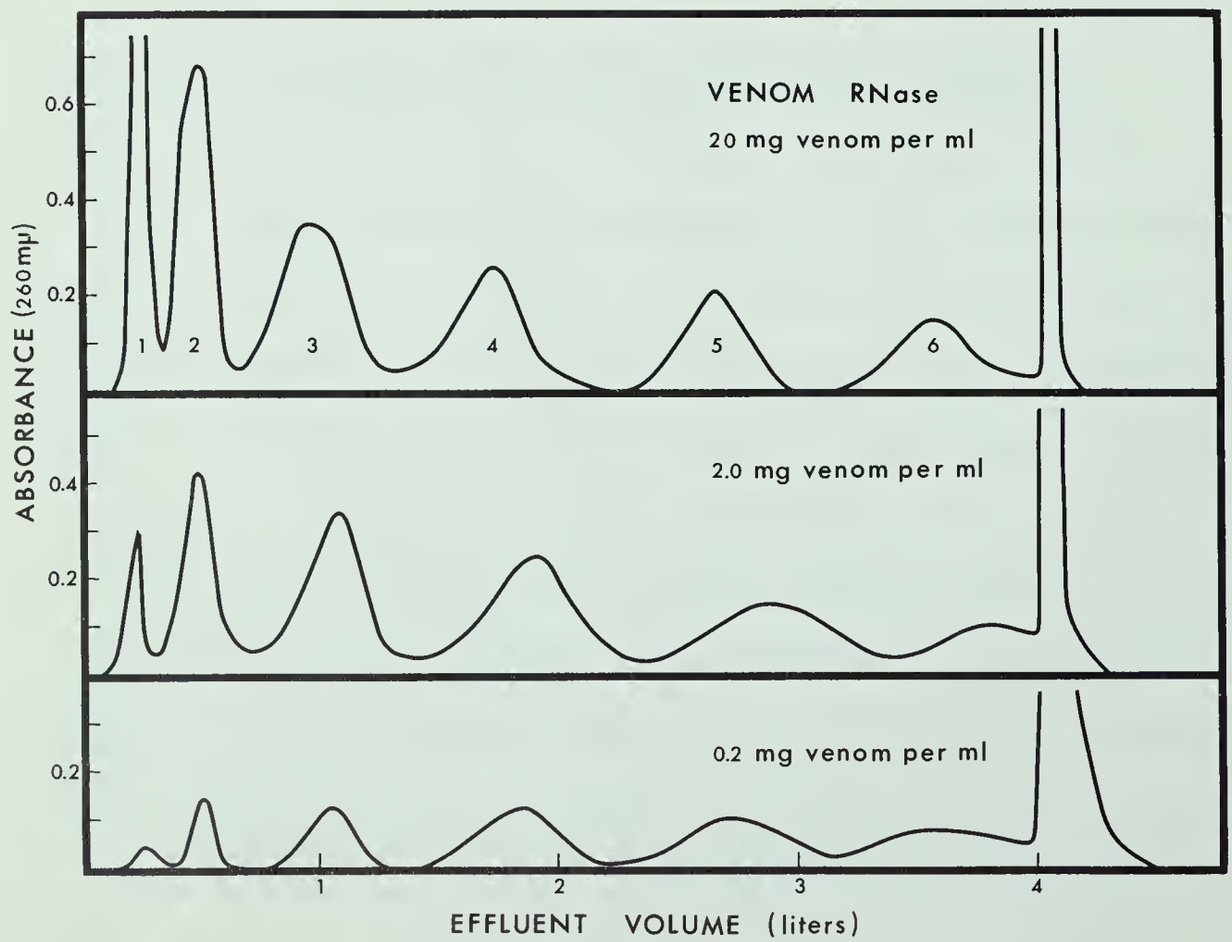


Figure 9



Legend of Figures 8 and 9

Absorbance profiles of DEAE-Sephadex fractionations of partial venom and pancreas RNase hydrolysates

At the highest concentration of enzyme (venom RNase, 20 mg/ml; pancreas RNase, 0.5 µg/ml) each peak consisted of a mixture of 'cyclic- and open-ended' isopliths in which the 'open-ended' isoplith contained one less nucleotide than the 'cyclic-ended' isoplith. For example, peak 2 consisted of NpN>p and Np compounds; peak 3 consisted of NpNpN>p and NpNp compounds. At the two lower concentrations of venom and pancreas RNase, the successive peaks consisted of predominantly 'cyclic-ended' isopliths of increasing chain length since there was very little nucleoside formed by alkali hydrolysis of the PMase-treated fractions. Subsequent paper chromatographic analysis of the hydrolysis products showed that there was some 'open-ended' material (less than 10%) produced at the lower enzyme concentrations.

The material eluted by the passage of four liters of eluent accounted for 84%, 53%, and 24% of the initial 700 s.u. charge in the case of the pancreas RNase digests at 0.5 γ, 0.05 γ and 0.005 γ RNase per ml digest, respectively. Similarly, in the case of the venom RNase, at 20 mg, 2 mg, and 0.2 mg venom per ml digest, the eluted material accounted for 81%, 62% and 32% of the initial charge. In all DEAE-Sephadex fractionations, the material remaining on the column after the passage of four liters of eluent was removed by elution with starting buffer containing one molar sodium chloride.

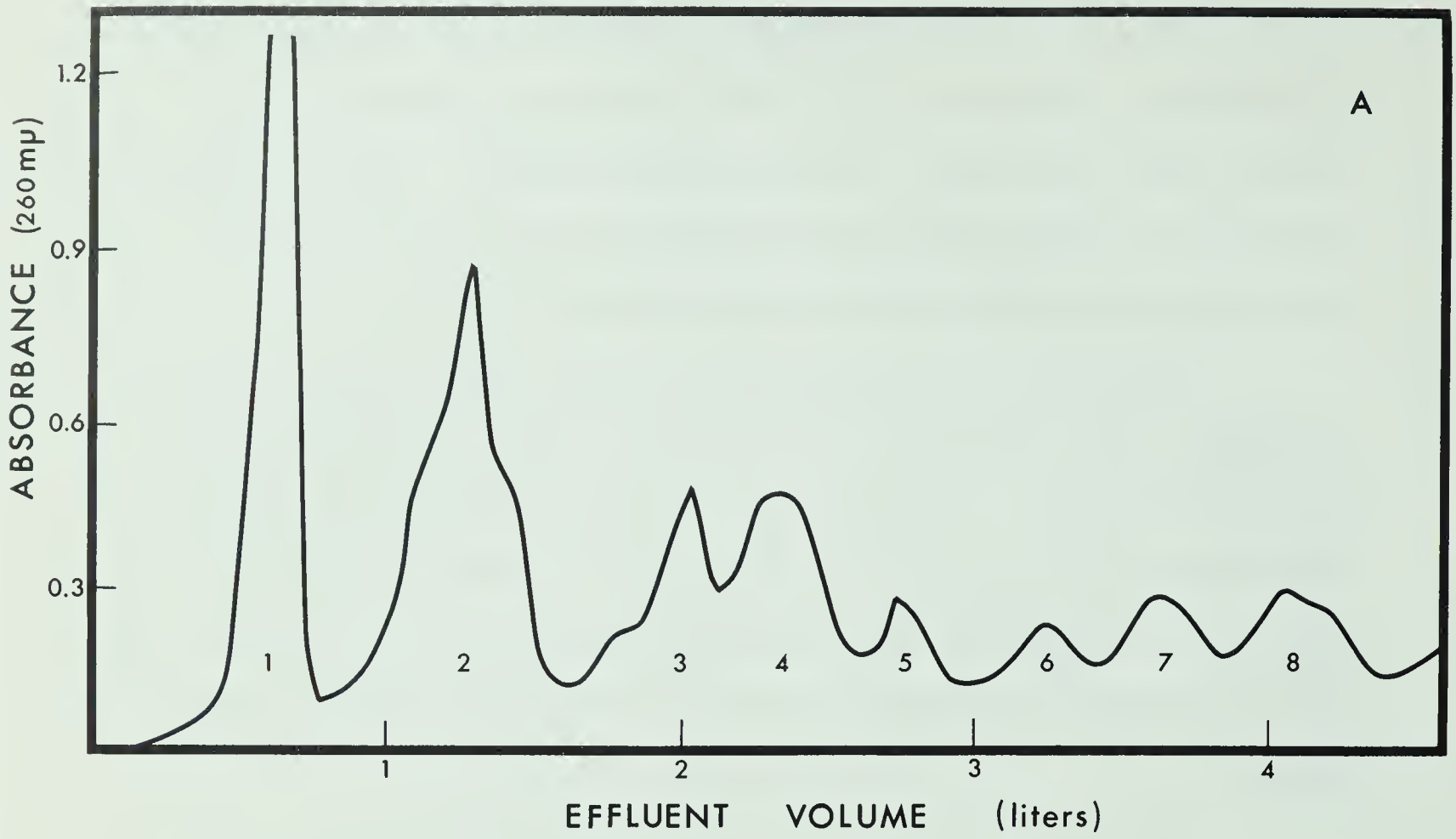
Specialties Co.) and a constant flow rate of 2 ml per minute was maintained with a micropump (Buchler Instruments). The absorbance spectrum of each fraction was measured with a Bausch and Lomb Model 505 Spectrophotometer. The absorbance profiles of DEAE-Sephadex fractionations of partial RNase hydrolysates of RNA can be seen in Figs. 8 and 9.

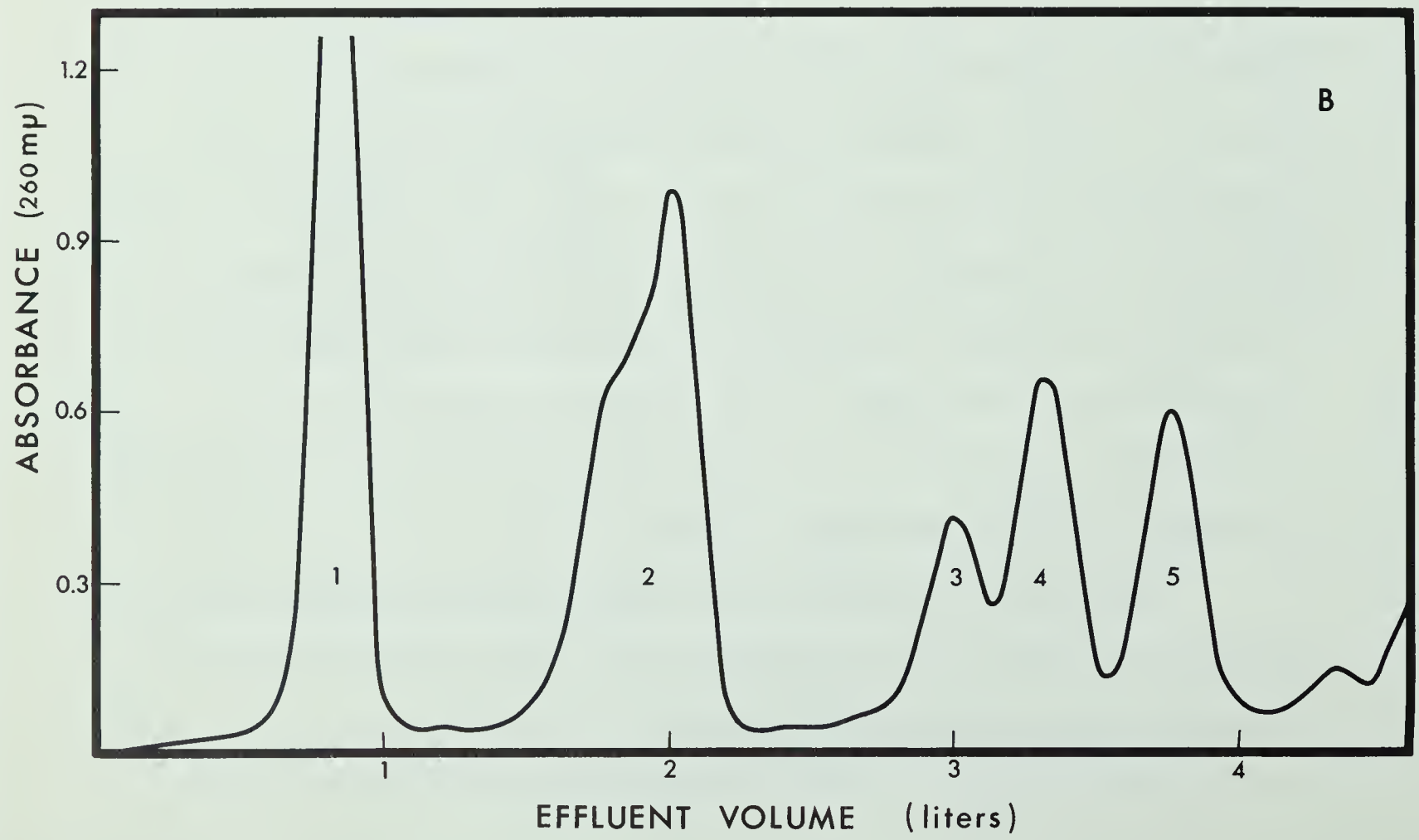
Fractions comprising each peak were pooled for desalting. The pools containing mono- and dinucleotides were adjusted to pH 5 and were freed of salt and urea by passage through a charcoal pad, using 50 mg of charcoal per 10 s.u. (260 mμ). When more than 100 mg of charcoal was required, it was mixed with an equal weight of celite to avoid slow flow rates.

The pools containing trinucleotides or larger oligonucleotides were freed of salt and urea by passage through DEAE-cellulose(carbonate). The oligonucleotide sample was diluted five-fold with 0.01 M ammonium carbonate and passed onto a 2.5 x 8 cm DEAE-cellulose(carbonate) column preequilibrated with 0.01 M ammonium carbonate. The column was washed with 100 ml of water, eluted with 200 ml of 0.01 M ammonium carbonate in order to remove chloride and then washed with copious amounts of water. The nucleate derivatives were eluted with 2 x 50 ml of 1 M pyridinium formate and recovered by evaporation as described in Part I.

Resolution of individual mononucleotides and dinucleotides was effected by two-dimensional paper chromatography using the systems described earlier (p. 17).

Figure 10


ABSORBANCE (260 mμ)
1.2
0.9
0.6
0.3
A
1
2
3
4
5
6
7
8
1
2
3
4
EFFLUENT VOLUME (liters)


ABSORBANCE (260 mμ)
1.2
0.9
0.6
0.3
B
1
2
3
4
5
1
2
3
4
EFFLUENT VOLUME (liters)

Legend of Figure 10

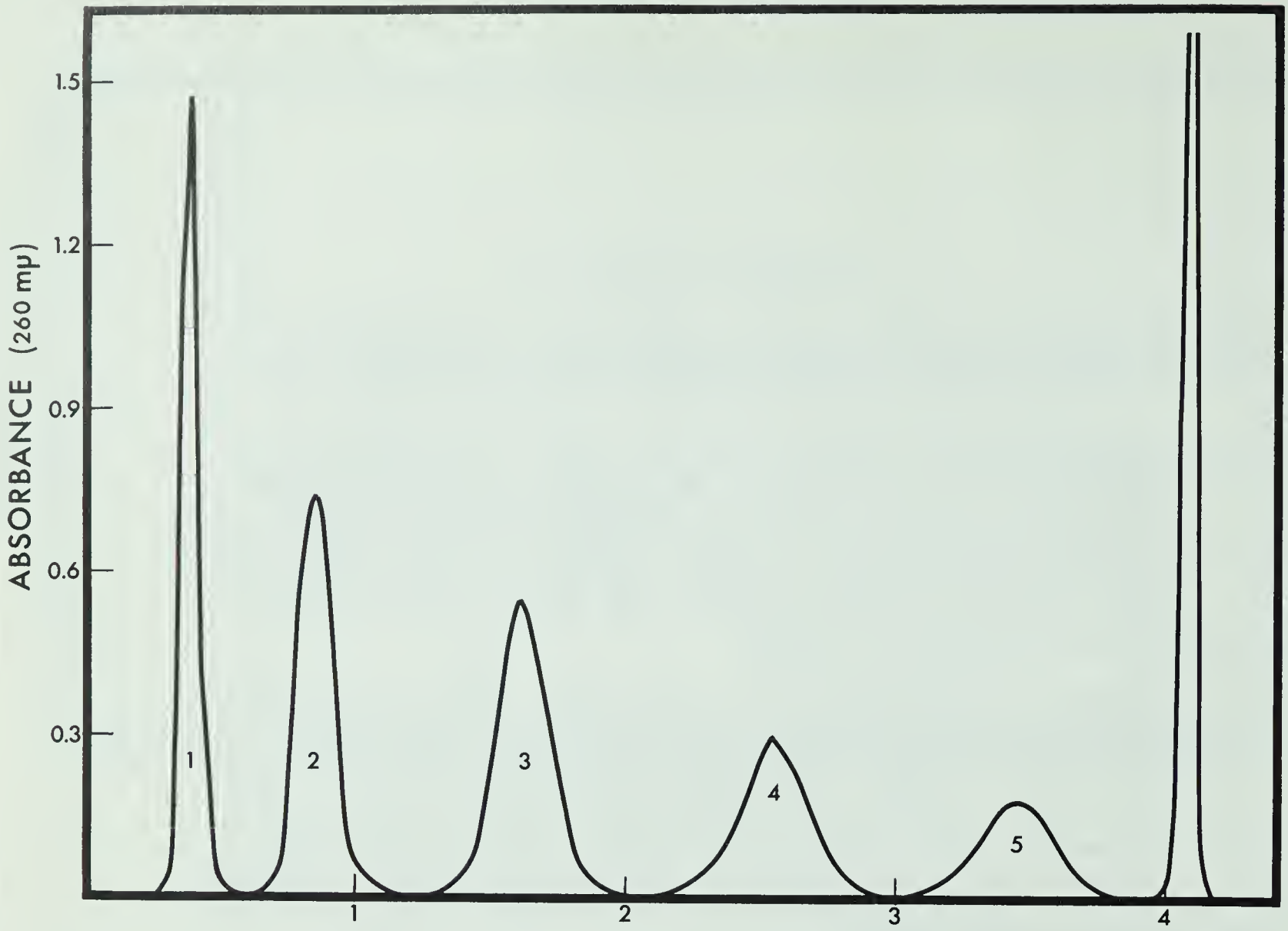
The absorbance profile of the fractionation of complete RNase hydrolysates on DEAE-Sephadex when solutions containing weak acid anions are employed as eluents.

(A) Fractionation of wheat embryo rRNA, pretreated with pancreas RNase (50 γ per ml digest), was conducted on a 60 x 25 cm column of DEAE-Sephadex(chloride) A-25 medium using a linear gradient of 0.4 M to 0.65 M TRIS formate (pH 7.8) in 8 M urea. After passage of 7 liters of the eluent, 80% of the initial 1525 s.u. charge was eluted.

After the removal of salt and urea, peak 1 was shown by paper chromatography to consist of pyrimidine mononucleotides. The remaining peaks were hydrolyzed in alkali, after the removal of salt and urea. From the ratio of purine and pyrimidine mononucleotides released after alkali hydrolysis, peak 2 was found to consist of dinucleotides, peak 3 was found to consist of di- and trinucleotides and peaks 4 to 8 consisted of trinucleotides.

(B) Fractionation of wheat embryo rRNA, pretreated with 50 γ RNase per ml digest, was performed on a 60 x 25 cm column of DEAE-Sephadex employing a linear gradient of 0.25 to 0.75 M sodium acetate in 0.01 M TRIS acetate buffer (pH 7.6) in 8 M urea. After passage of 7 liters of eluent, 90% of the initial 1480 s.u. charge was eluted. Since it was apparent that peaks beyond peak 2 were not cleanly separated, the isoplith content of the peaks was not analyzed.

Figure 11



The absorbance profile of the fractionation of complete RNase hydrolysates on DEAE-Sephadex when sodium chloride solution is employed as eluent.

The fractionation was performed as described in the text. Peaks 1 to 5 consisted of mononucleotides to pentanucleotides, respectively, and accounted for 82% of the initial charge. The nucleate material remaining on the column, after the passage of four liters of eluent, was removed by starting buffer containing one molar sodium chloride.

(c) Factors influencing the resolution of oligonucleotides on DEAE-Sephadex.

Since the removal of nonvolatile salts such as sodium chloride from oligonucleotide fractions presents several technical problems, attempts were made, without success, to devise a fractionation scheme which would employ buffers whose anions could be directly converted to volatile salts, while still effecting adequate separation of the oligonucleotide mixtures.

Toward this end, attempts were made to effect elution of oligonucleotides from DEAE-Sephadex by weak acid anions such as formate and acetate in the hope that subsequent desalting on DEAE-carbonate and elution with ammonium carbonate to remove the chloride anion could be replaced by the customary desalting procedure using DEAE-formate and pyridinium formate elution.

The elution patterns of Fig. 10 show that the mononucleotides and dinucleotides, formed by exhaustive hydrolysis of RNA with pancreas RNase are eluted as reasonably discrete peaks but that discrete peaks corresponding to higher oligomers are not apparent when elution is effected with weak acid anions. By contrast, discrete peaks are obtained with higher oligomers in the elution pattern produced by the chloride anion (Fig. 11). It is perhaps noteworthy in regard to the poor resolution obtained with the weak acid anions in these experiments, that Bartos et al. reported having used only weak acid anions in their attempts to achieve separation on the basis of net charge in the case of T_1-RNase digests on DEAE-cellulose. It would seem important to establish that the

TABLE X

Mononucleotide Analysis of a PDase Hydrolysate of Wheat Embryo sRNA and rRNA

Component	mole(s)/100 mole nucleotide	
	sRNA	rRNA
pA	19.8	23.2
pAx	0.005	0.54
plMeA	0.18	-
pN^6MeA	0.73	-
pG	29.3	30.1
pGx	0.47	0.38
plMeG	0.73	-
p7MeG	0.18	-
pN^2MeG	0.29	-
pN^2,N^2diMeG	0.55	-
pI	0.090	-
pC	26.3	24.8
pCx	0.40	0.35
p5MeC	1.37	-
pU	15.7	18.1
pUx	0.30	0.47
p5MeU	0.64	-
pΨ	2.7	1.8
pΨx	0.12	-

different elution behaviour observed with DEAE-cellulose and DEAE-Sephadex is not ascribable to the fact that weak acid anions were used with DEAE-cellulose whereas chloride was used with DEAE-Sephadex in attempts to resolve the products of T_1-RNase digestion.

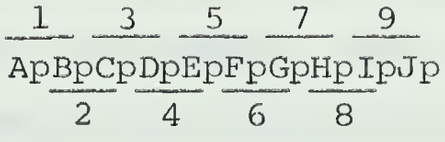
(iii) Theoretical Considerations Relating to the Cleavage of RNA by RNases.

The results of quantitative analyses for the mononucleotides produced by PDase hydrolysis of wheat embryo rRNA and sRNA are shown in Table X. The data of Table X can be used to calculate the amount of any individual oligonucleotide sequence per 100 moles of its isoplithic sequences in the RNA by presuming a random distribution of mononucleotides among the isopliths.

For purposes of calculating random proportions, the mole % values for the mononucleotides are converted to decimal fractions (or frequencies). The frequencies of pA and pG in wheat embryo rRNA are 0.232 and 0.301, respectively. If the mononucleotides are randomly distributed among all possible dinucleotide sequences, the frequency of the sequence ApG would be 0.232 x 0.301 = 0.070, i.e. ApG would account for 7.0 moles per 100 moles of total dinucleotide sequences in wheat embryo rRNA.

The observed amount of a given oligonucleotide sequence must be expressed as mole(s) per 100 moles of mononucleotides in comparing measured quantities with the amounts calculated for random distribution of mononucleotides among isopliths. Thus, if the observed amount of ApG is 7.0 moles per 100 moles

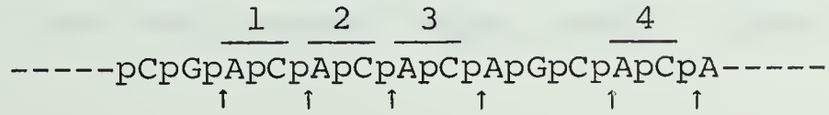
of mononucleotides, then ApG accounts for 14.0 mole % of the total mononucleotides in the RNA since there are 2 moles of mononucleotide per mole of dinucleotide; however, ApG accounts for only 7.0 moles per 100 moles of dinucleotide sequences in the RNA because the number of dinucleotide sequences is the same as the number of mononucleotide residues in a ribonucleate chain. It can be seen from the following example that even for a decanucleotide, there are nearly as many dinucleotide sequences as there are mononucleotide residues.



It is apparent that there are 9 dinucleotide sequences per 10 mononucleotides since each non-terminal mononucleotide participates in 2 dinucleotide sequences. For sRNA chains, which are about 80 nucleotides long, there are 79 dinucleotide sequences and for rRNA chains that are about 1300 nucleotides long, there are 1299 dinucleotide sequences. Thus, it is a good approximation to neglect the fact that terminal mononucleotides participate in only a single dinucleotide sequence because the high degree of polymerization (and small percentage of chain terminals) in naturally occurring RNA makes the mole(s) of a particular dinucleotide sequence per 100 moles of total dinucleotide sequences nearly equal to the mole(s) of the dinucleotide per 100 moles of mononucleotides. A similar situation would obtain with longer chain isopliths but the approximation becomes less tenable with increasing chain length (i.e., only 76 pentanucleotide sequences, 75 hexanucleo-

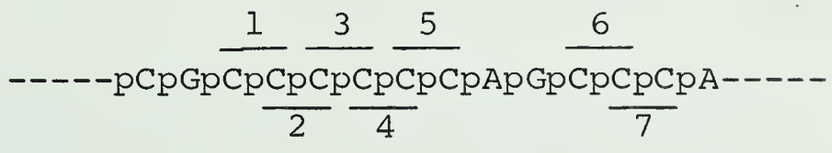
tide sequences, 74 heptanucleotide sequences, etc. per 80 moles mononucleotides) until ultimately, of course, there would only be one octadecanucleotide sequence per 80 mononucleotides for a homogeneous preparation of sRNA. Because of the greater number of sequences in a heterogeneous preparation of sRNA chains (20 different species would give 20 different octadecanucleotide sequences), all approximations to randomness would be expected to have greater validity with increasing heterogeneity. For similar reasons, the approximation is increasingly valid for RNA of greater chain length since, for example, rRNA would have nearly 1300 dinucleotide sequences per mole whereas sRNA would have only about 80 dinucleotide sequences per mole. Calculations made in the present work are confined to di- and trinucleotide sequences in heterogeneous preparations and the approximation mentioned above would be expected to be valid within the limits of accuracy of the measured values.

The feasibility of direct measurement of the amount of a given dinucleotide sequence per 100 moles of total dinucleotide sequences is dependent not only on the availability of a specific degradation procedure but also upon whether or not the sequence is homologous or heterologous. In the case of a heterologous sequence, it is possible, in principle, to recover all of the sequence present in an RNA specimen if a suitable degradation procedure is available. This is illustrated below using ApC as an example.



If the cleavages indicated could be specifically induced without cleavage between Ap and C, then all ApC sequences could be recovered; however, such degradation methods are not available except in the case of 2'-O-methyl substituted sequences where all of the AxpC sequences can be recovered by alkali hydrolysis of RNA. It is, however, possible to recover part of the ApC sequences in RNA chains by complete hydrolysis with pancreas RNase. Thus, it is possible to compare the measured amount of (Pyp)ApC sequences with the amount predicted for a random distribution of mononucleotides among all possible trinucleotide sequences.

In the case of a homologous sequence, it is not possible, even in principle, to recover <u>all</u> of the sequence in an RNA specimen for comparison with a random distribution calculation. This is illustrated below for CpC.



There are 7 CpC sequences in this segment of an RNA chain but it would only be possible to isolate 4 of these as physical entities because 3 of the sequences arise from the fact that more than two Cp residues are contiguous. Thus, it would not be possible to isolate the total amount of CpC sequences for comparison with statistical predictions of the <u>total</u> amount of CpC sequences in an RNA specimen.

It is possible, however, to classify dinucleotide sequences into four groups: PupPu, PupPy, PypPu, PypPy, and

TABLE XI

Relative Amounts of Dinucleotide Sequences in sRNA and rRNA.

Dinucleotide Sequence	Calculated mole(s)/100 moles of total Dinucleotide Sequences
rRNA:	
PypPy	$(.248 + .181)^2 = 0.184 \times 100 = 18.4$
PypPu	$(.248 + .181)(.232 + .301) = 0.229 \times 100 = 22.9$
PupPy	$(.232 + .301)(.248 + .181) = 0.229 \times 100 = 22.9$
PupPu	$(.232 + .301)^2 = 0.284 \times 100 = 28.4$
sRNA:	
PypPy	$(.263 + .157)^2 = 0.176 \times 100 = 17.6$
PypPu	$(.263 + .157)(.198 + .293) = 0.206 \times 100 = 20.6$
PupPy	$(.198 + .293)(.263 + .157) = 0.206 \times 100 = 20.6$
PupPu	$(.198 + .293)^2 = 0.241 \times 100 = 24.1$

measure the total amounts of PypPu and PypPy sequences by indirect means. Each of the four groups accounts for 4 of the 16 possible dinucleotide sequences which can arise from different arrangements of the four major mononucleotides in RNA. The PypPu and PypPy sequences account for 8 of the 16 sequences, and are susceptible to pancreas RNase, while the remaining 8 are accounted for by PupPu and PupPy sequences, which are not susceptible to pancreas RNase. The relative amounts of the different groups of dinucleotide sequences have been calculated for wheat embryo rRNA and sRNA presuming a random distribution of the four mononucleotides among different dinucleotide sequences (Table XI).

Since pancreas RNase hydrolyzes all PypPu and PypPy linkages in RNA chains to yield pyrimidine mononucleotides and a series of oligonucleotides, having the general structure $(Pup)_nPyp$, it is possible to measure experimentally the total amounts of the PypPu and PypPy linkages in a ribonucleate specimen by end group and mononucleotide analysis of the products produced by complete hydrolysis of the RNA by pancreas RNase.

Thus, the Pu portion of every PypPu sequence will appear as a terminal group of an oligonucleotide having the general structure $(Pup)_nPyp$, and conversely, every terminal Pu residue of the $(Pup)_nPyp$ oligonucleotides will have derived from the cleavage of a PypPu linkage. The mole % of Pu nucleoside released from terminal residues by PDase digestion of the hydrolysis products, formed by complete RNase hydrolysis of RNA, will therefore correspond to the moles of

PypPu dinucleotide sequences per 100 moles of dinucleotide sequences because there is one mole of Pu per mole of PypPu sequence.

For complete hydrolysis by RNase, the mole % of Pyp mononucleotides will correspond to the quantity of PypPy sequences per 100 moles of dinucleotide sequences because the sequences are homologous. It can be seen below that each PypPy sequence will give rise to a single Pyp mononucleotide after complete RNase digestion.

1 sequence: ---PypPyp--------- ⟶ 1 Pyp + fragments
↑ ↑

2 sequences: ---PypPypPyp------ ⟶ 2 Pyp + fragments
↑ ↑ ↑

3 sequences: ---PypPypPypPyp--- ⟶ 3 Pyp + fragments
↑ ↑ ↑ ↑

In this way, it is possible to compare the observed proportions of the PypPu and PypPy linkages with the calculated amounts based on a random distribution of mononucleotides among dinucleotide sequences.

Furthermore, since every PypPu linkage broken by partial RNase digestion will appear as a Pu nucleoside after PDase digestion of the RNase hydrolysis products, the proportion of the total PypPu linkages cleaved in a partial hydrolysate will be given by:

$$\frac{\text{(Pu nucleosides formed by PDase digestion of the products of partial hydrolysis by the RNase)}}{\text{(Pu nucleosides formed by PDase digestion of the products of complete hydrolysis by pancreas RNase)}} \times 100$$

Since all PupN linkages are not susceptible to pancreas RNase, and the proportion of terminal PypPu sequences in RNA is negligible, all Pu parts of PypPu sequences remain part of oligonucleotide fragments after complete or partial RNase digestion of RNA.

The measurement of Py nucleosides after PDase digestion of the partial hydrolysis products produced by the RNase can, however, only provide a minimal estimate of the endonucleolytic scission of PypPy linkages since some 3'-linked Py terminals of oligonucleotides are released by exonucleolytic cleavage. Thus, the minimal proportion of PypPy linkages cleaved in a partial RNase hydrolysate will be given by:

$$\frac{\text{(Py nucleosides formed by PDase digestion of the products of partial hydrolysis by the RNase)}}{\text{Pyp mononucleotides formed by complete RNase digestion}} \times 100$$

The total proportion of PypPy linkages cleaved by partial RNase hydrolysis can be obtained by summing the minimal proportion of endonucleolytic PypPy scissions and the proportion of total mononucleotides released by partial RNase digestion since each PypPy cleavage, not accounted for as a 3'-linked terminus of a fragment, will be accounted for by the release of a pyrimidine mononucleotide after RNase hydrolysis. This can be seen to be the case by examining the exonucleolytic release of Pyp mononucleotides.

Release from the 5'-linked terminus of a fragment by RNase clearly corresponds to the cleavage of a PypPyp linkage.

i.e. -----PypPyp $\longrightarrow$ -----Pyp + Pyp

TABLE XII

End Group Analysis of the Products Formed by Complete Hydrolysis of Wheat Embryo rRNA by Pancreas RNase.

Analysis	Nucleosides			Nucleoside Diphosphates		
	A	G	Total	pCp	pUp	Total
	mole(s)/100 moles mononucleotides			mole(s)/100 moles mononucleotides		
1	6.64	8.77	15.4	-	-	-
2	2.62	2.96	5.58	5.59	4.81	10.4
3	0.50	0.53	1.03	0.66	0.37	1.03
	9.76	12.2	22.0	6.25	5.18	11.4

In this, as in subsequent tables, the values are quoted to 3 figures with the understanding that the measured quantities are probably accurate to within ±5%.

TABLE XIII

Proportions of PypPu Linkages in Wheat Embryo rRNA.

Linkage	Measured	Calculated
	moles/100 moles mononucleotides	
PypA	9.8	10.0
PypG	12.2	12.9
PypPu	22.0	22.9

Release from the 3'-linked terminus of a fragment having a subterminal Py or Pu residue also corresponds to the cleavage of a PypPy linkage:

i.e. PypPyp----- ⟶ Pyp + Pyp-----
↑

PypPup----- ⟶ Pyp + Pup-----
↑

The released Pyp mononucleotide, in both cases, is a measure of the PypPy cleavage, which had temporarily left the released Pyp nucleotide as the 3'-linked terminus of an oligonucleotide.

(iv) Cleavage of Wheat Embryo rRNA by Venom and Pancreas RNases.

(1) Complete hydrolysis of rRNA by pancreas RNase.

(a) The quantity of PypPu linkages in wheat embryo rRNA.

The end group analysis of fragments produced by complete RNase hydrolysis of rRNA is shown in Table XII. The nucleosides measured in analysis 1 were those recovered after PDase hydrolysis of the products resulting from the complete RNase digestion of wheat embryo rRNA. This treatment left an oligonucleotide residue, which was eluted along with the nucleoside diphosphates, from DEAE-cellulose. The mixture of oligonucleotides and pNp compounds was treated with PMase in analysis 2 and the products passed through DEAE-cellulose. The pyrimidine nucleosides derived from pNp compounds amounted to about 65% of the purine nucleosides found in analysis 1.

TABLE XIV

Hyperchromicity in RNase Hydrolysates.

Enzyme Concentration per ml Digest	% Hyperchromicity* 1 hr	24 hrs
0.005γ pancreas RNase	3.4%	16%
0.05 γ " "	6.7%	24%
0.5 γ " "	16.3%	25%
50. γ " "	26. %	27%
0.25 ml snake venom PDase	-	42%

*Measured at 260 mμ in 0.25 M TRIS formate, pH 7.6.

This might indicate that the oligonucleotide residue of analysis 1 contained compounds of the type $(pPu)_nPyp$ as the result of a disproportionately greater release of nonphosphorylated terminals than of phosphorylated terminals in the PDase digestion in analysis 1 (Felix et al., 1960). Alternatively, and more probably, there may have been substantial destruction of pNp compounds because of the high PDase concentration and relatively low pN concentration in the PDase digest of analysis 1. The oligonucleotides were treated for 60 seconds with 1 M alkali to inactivate PMase and then hydrolyzed with PDase to release 3'-linked terminal purine nucleosides. The contribution of the terminals from residual PupPyp compounds was included as analysis 3 in Table XII. The residue remaining amounted to only 2% of the initial RNA, and since it contained only oligonucleotides larger than dinucleotides, it could not contribute more than 0.7 m % to the end group analysis.

The amounts of PypA, PypG and PypPu linkages, based on the data of Table XII, are compared in Table XIII with the amounts of these linkages calculated for random distribution of mononucleotides in different dinucleotide sequences.

(b) The quantity of PypPy linkages in wheat embryo rRNA.

To relate the molar quantities of the mono- and dinucleotides to the total nucleotides in hydrolyzed RNA samples, hyperchromicity following hydrolysis must be assessed. The values for hyperchromicity after varying degrees of hydrolysis by pancreas RNase are shown in Table XIV along with the

TABLE XV

Mononucleotide and Dinucleotide Analyses of the Products Formed by Complete Hydrolysis of Wheat Embryo rRNA by Pancreas RNase.

Compound	moles/100 moles mononucleotides
Cp	10.7
Up	7.0
ApCp	1.6
ApUp	1.5
GpCp	3.3
GpUp	2.1

TABLE XVI

Proportions of PypPy and PypPupPy Sequences in Wheat Embryo rRNA.

Sequence	Measured	Calculated
	moles/100 moles mononucleotides	
PypPy	17.7	18.4
PypApC	1.6	2.5
PypApU	1.5	1.8
PypGpC	3.3	3.2
PypGpU	2.1	2.3

TABLE XVII

Recoveries of Nucleate Derivatives from DEAE-Sephadex Column Effluents.

RNase Concentration per ml Digest	Peak 1 %	Peak 2 %	Peak 3 %
Pancreas:			
0.5 γ	45(75)	59(90)	70(90)
0.05 γ	40(72)	74(98)	72(97)
0.005 γ	21(92)	66(99)	51(92)
Venom:			
20 mg	55(75)	71(98)	51(61)
2 mg	34(63)	75(100)	43(64)
0.2 mg	27(37)	81(100)	48(63)

s.u. recovered after paper chromatography relative to s.u. in column fraction.

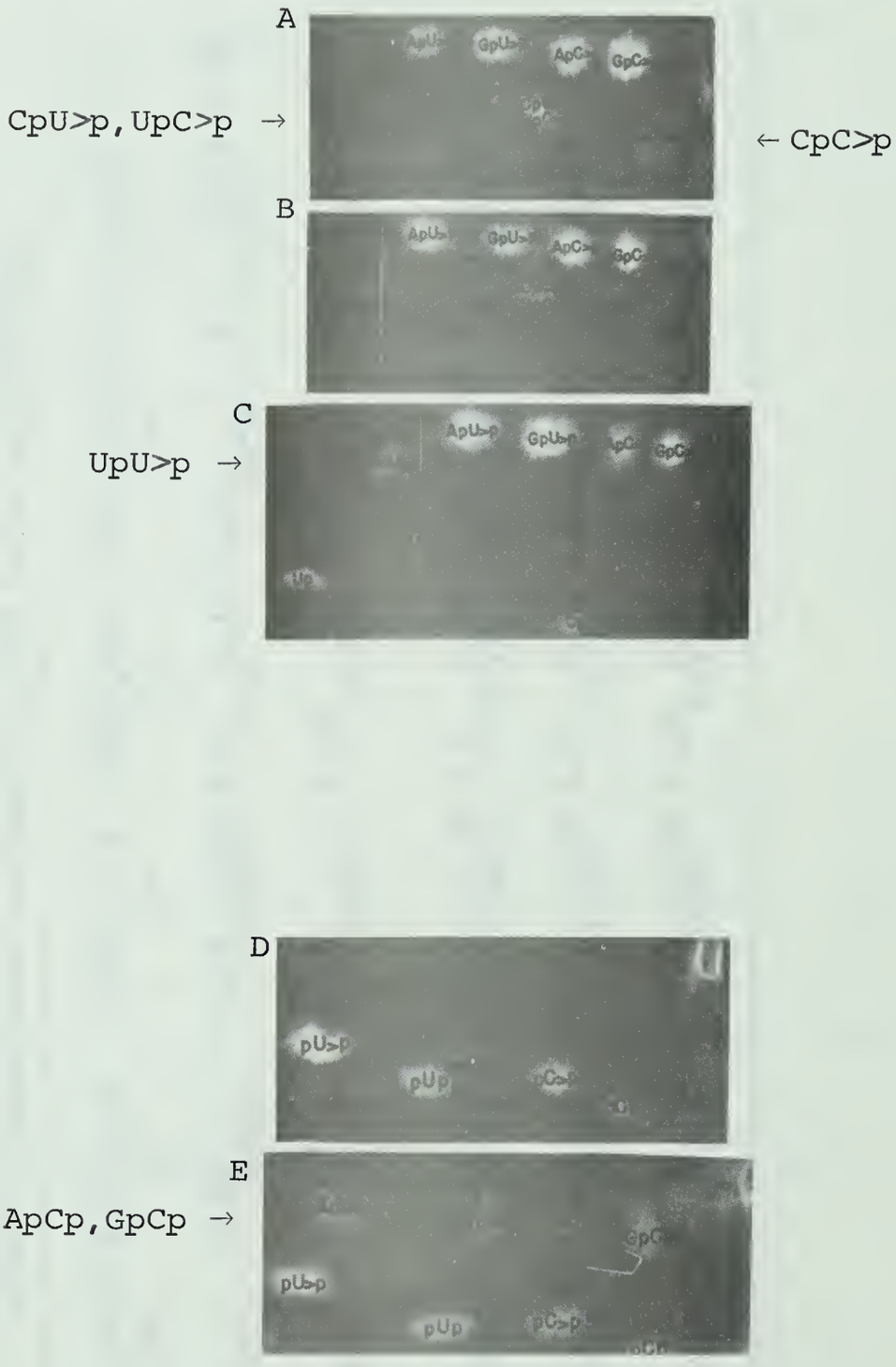
Figures in parentheses = s.u. recovered after desalting relative to s.u. in column fraction.

TABLE XVIII

Mononucleotide and Dinucleotide Analysis of the Products Formed by Partial Hydrolysis of Wheat Embryo rRNA by Pancreas and Venom RNases.

RNase Concentration per ml digest	U>p	Up	C>p	Cp	ApC>p	ApCp	GpC>p	GpCp	ApU>p	ApUp	GpU>p	GpUp	UpC>p	CpC>p	UpU>p	Total
	mole(s) constituent nucleotides/100 moles nucleotides															
Pancreas:																
0.5 γ	3.2	0.72	1.6	1.1	0.58	1.8	0.68	4.4	0.93	0.84	1.5	1.4	0.20	-	0.38	
	3.92		2.7		2.4		5.1		1.8		2.9					19.4
0.05 γ	0.62	-	0.82	-	1.16	0.29	1.7	0.17	0.75	-	1.1	0.07	0.88	0.38	0.20	
	0.62		0.82		1.45		1.9		0.75		1.2					8.2
0.005 γ	0.04	-	0.06	-	0.50	-	0.36	-	0.35	-	0.32	-	0.15	0.08	0.04	
	0.04		0.06		0.50		0.36		0.35		0.32					1.9
Venom:																
20 mg	3.1	1.7	2.3	0.83	1.86	0.18	3.2	0.52	0.96	0.46	1.3	1.3	0.76	0.62	0.30	
	4.8		3.13		2.04		3.72		1.42		2.6					19.4
2 mg	0.41	0.29	0.20	-	1.0	0.26	1.1	0.27	1.1	0.12	0.83	0.31	0.93	0.68	0.31	
	0.70		0.20		1.26		1.37		1.22		1.14					7.8
0.2 mg	0.07	-	0.07	-	0.59	0.04	0.18	0.03	0.64	0.01	0.24	-	0.17	0.18	0.10	
	0.07		0.07		0.63		0.21		0.65		0.24					2.3

Figure 12



Legend of Figure 12

Ultraviolet contact photographs of typical two-dimensional paper chromatograms showing the resolution of (i) Np and NpN>p compounds, and (ii) NpNp, pNp and pN>p compounds. The origin of the chromatogram is, in each case, at the upper right-hand corner of the photographs. Development of the chromatograms, in relation to the photos, was from right to left (first dimension), and from top to bottom (second dimension).

A, B and C depict the separation of Np and NpN>p compounds produced by digestion of wheat embryo rRNA for 24 hrs. with pancreas RNase.

A - 0.005 µg enzyme per ml of digest,

B - 0.05 µg enzyme per ml of digest,

C - 0.5 µg enzyme per ml of digest.

The compounds were eluted together in the second peak of DEAE-Sephadex column fractionations of the RNase digests (cf. Figure 8).

D and E depict the resolution of PupPyp, pPyp and pPy>p compounds formed by PDase digestion of the products of partial venom RNase digestion of wheat embryo rRNA.

D - 2 mg acid-treated venom per ml of RNase digest,

E - 20 mg acid-treated venom per ml of RNase digest.

The compounds were eluted together in DEAE-cellulose fractionations of PDase digests.

hyperchromicity found for complete conversion to mononucleotides by venom PDase. Thus, the s.u. in 24 hour digests of rRNA by pancreas RNase were multiplied by $\frac{1.42}{1.27} = 1.12$ (50 γ RNase), $\frac{1.42}{1.25} = 1.14$ (0.5γ RNase), $\frac{1.42}{1.24} = 1.15$ (0.05γ RNase), and $\frac{1.42}{1.16} = 1.22$ (0.005γ RNase) before dividing by 11 to obtain the micromoles of nucleotide material in the digest analyzed for mono- and dinucleotides.

The mononucleotide and dinucleotide analysis of the products formed by complete pancreas RNase hydrolysis is shown in Table XV. The components were separated by direct two-dimensional paper chromatography and the recovery of ultraviolet absorbing material was 95-100%.

The measured proportions of PypApC, PypApU, PypGpC and PypGpU sequences are compared with the statistically random proportions in Table XVI.

(2) Partial hydrolysis of wheat embryo rRNA by venom and pancreas RNases.

(a) Release of mononucleotide and dinucleotide end products.

The data summarized in Table XVII show the recovery of s.u. from each column fraction after elution of compounds from paper chromatograms. The values shown in Table XVIII have not been corrected for losses incurred during recovery from column fractions or for hyperchromicity of dinucleotides, and are thus minimal values. Ultraviolet contact prints of chromatograms showing the separation of dinucleotides and mononucleotides recovered from peak 2 of DEAE-Sephadex columns are shown in Fig. 12.

TABLE XIX

The Proportional Release of Mononucleotides and Dinucleotides by Partial Venom RNase Hydrolysis of rRNA.

Compound	RNase Concentration per ml Digest		
	0.20 mg	2.0 mg	20 mg
C>p + Cp	0.65	1.9	29
U>p + Up	1.0	10	69
ApC>p + ApCp	20	39	64
ApU>p + ApUp	22	41	47
GpC>p + GpCp	3.3	21	56
GpU>p + GpUp	5.7	27	62

The calculations were based on the actual quantities recovered and are therefore minimal values.

TABLE XX

The Proportional Release of Mononucleotides and Dinucleotides by Partial Pancreas RNase Hydrolysis of rRNA.

Compound	RNase Concentration per ml Digest		
	0.005γ	0.05γ	0.5γ
C>p + Cp	0.28	7.7	25
U>p + Up	0.29	8.9	56
ApC>p + ApCp	16	46	75
ApU>p + ApUp	12	25	60
GpC>p + GpCp	5.4	28	77
GpU>p + GpUp	7.6	26	69

The calculations were based on the actual quantities recovered and are therefore minimal values.

TABLE XXI

End Group Analysis of the Products Formed in 24 Hours by Partial Hydrolysis of Wheat Embryo rRNA by Venom and Pancreas RNases.

RNase Concentration per ml Digest	A	G	C	U	Total	pC>p	pCp	pU>p	pUp	Total	% PypPu Linkages Cleaved	% PypPy Linkages** Cleaved
	mole(s)/100 moles nucleotides					mole(s)/100 moles nucleotides						
Pancreas:												
50γ	9.8	12.2	-	-	22.0	-	6.25	-	5.2	11.4	100	100
0.5	7.0	11.2	-	-	18.2	1.5	6.4	3.0	6.1	17.0		
	1.6	3.2	-	-	4.8*	-	3.7	-	1.0	4.7*		
	8.6	14.4			23.0	1.5	10.1	3.0	7.1	21.7	104	43
0.05	7.6	9.4	1.6	0.9	19.5	7.2	1.7	7.2	2.5	18.6		
	0.13	0.28	-	-	0.41*	-	0.42	-	-	0.42*		
	7.7	9.7	1.6	0.9	19.9	7.2	2.1	7.2	2.5	19.0	79	29
0.005	6.8	3.9	1.0	0.6	12.3	4.8	1.3	4.1	1.6	11.8	49	10
Venom:												
20 mg	6.9	11.0	1.7	0.7	20.3	4.7	6.2	3.0	5.8	19.7		
	0.8	1.6	-	-	2.4*	-	1.0	-	1.2	2.2*		
	7.7	12.6	1.7	0.7	22.7	4.7	7.2	3.0	7.0	21.9	93	66
2	8.1	7.2	3.4	1.7	20.4	5.4	2.7	6.0	4.1	18.2		
	-	0.3	-	-	0.3*	-	0.2	-	0.1	0.3*		
	8.1	7.5	3.4	1.7	20.7	5.4	2.9	6.0	4.2	18.5	71	35
0.2	7.2	2.3	1.4	1.1	12.0	3.2	1.2	4.6	2.2	11.2	43	15

*From dinucleotide residues.
**Assuming 75% recovery of Pyp mononucleotides.

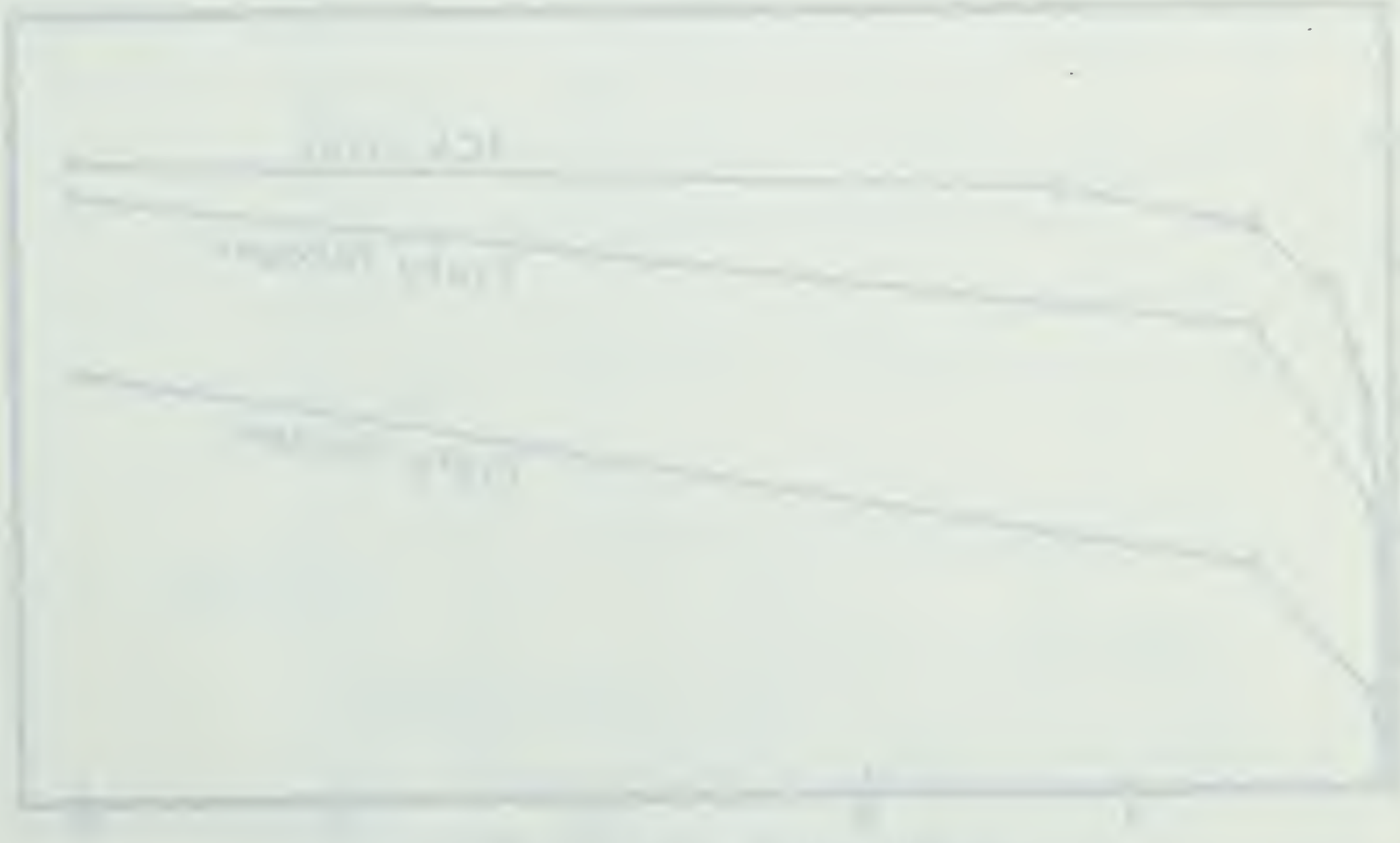
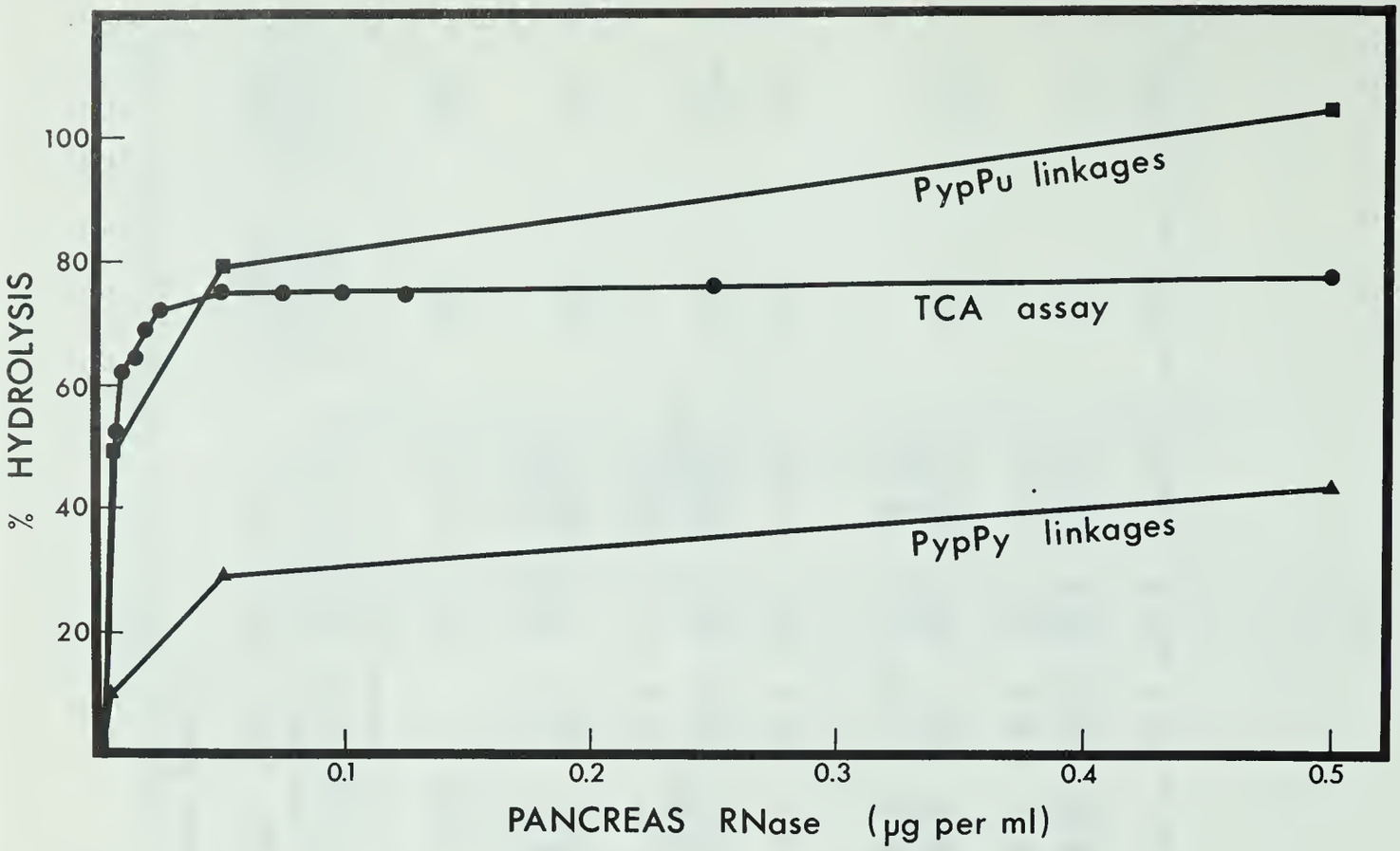
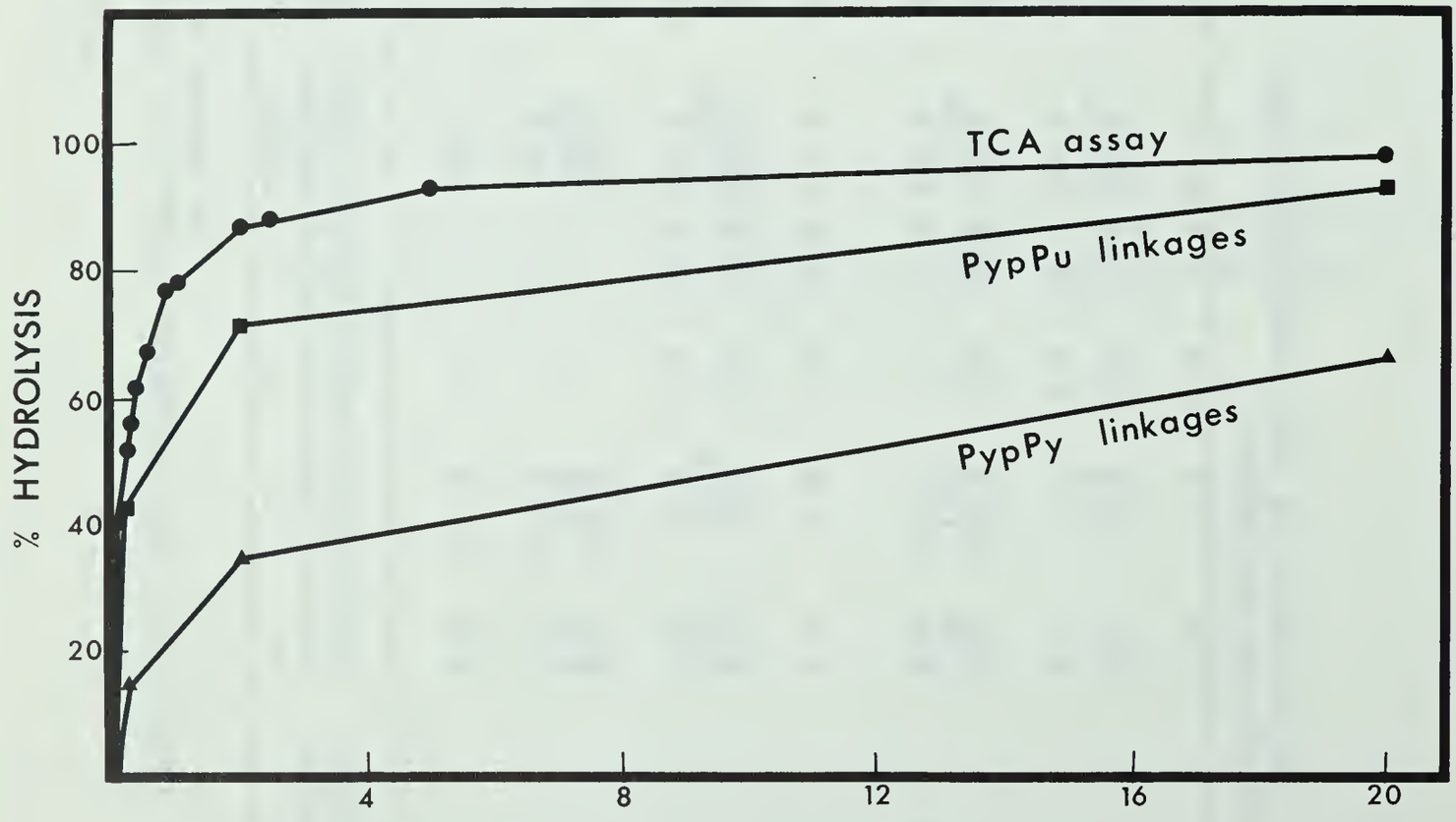


Figure 13





The rates of hydrolysis of PypPu linkages and PypPy linkages by venom and pancreas RNases

TABLE XXII

End Group Analysis of the Products Formed by the Partial Hydrolysis of Wheat Embryo rRNA by Venom and Pancreas RNases.

[RNase] per ml Digest	RNase Activity in TCA Assay	A	G	C	U	Total	pC>p	pCp	pU>p	pUp	Total	PypPu Linkages Cleaved
		mole(s)/100 moles nucleotides					mole(s)/100 moles nucleotides					
Venom, 24 hours hydrolysis												
0.6 mg	72%	6.0	3.3	1.6	1.2	12.1	4.3	0.41	5.6	1.4	11.7	42%
0.24	58	5.7	1.9	1.1	0.70	9.4	3.9	0.68	4.9	1.3	10.8	34
0.12	29	5.8	1.3	0.67	0.57	8.3	3.6	0.53	4.2	1.0	9.3	32
0.024	6	3.2	0.24	0.25	0.25	3.9	1.3	0.12	2.1	0.44	4.0	16
Venom, 1 hour hydrolysis												
20.0 mg*	66%	7.3	5.2	2.0	2.2	16.7	5.4	1.0	5.2	3.8	15.4	57%
1.2	37	5.4	0.71	0.58	0.29	7.0	3.1	0.59	2.9	0.51	7.1	28
0.6	29	5.0	0.59	0.44	0.38	6.4	2.6	0.52	2.7	0.84	6.7	25
0.072	5	1.51	0.057	0.10	0.050	1.7	0.48	0.15	0.89	0.27	1.8	7
0.024	2	0.72	0.080	0.056	0.12	0.98	0.29	0.14	0.49	0.17	1.1	4
Pancreas, 1 hour hydrolysis												
0.03 γ	32%	5.7	2.32	0.76	0.40	9.2	3.4	0.70	3.4	0.81	8.3	37%
0.0006	0.2	0.86	0.16	0.15	0.054	1.2	0.52	0.045	0.48	0.078	1.1	5

*Dialyzed stock solution (80 mg venom/ml) used in the experiments shown in Table XXI. All other venom RNase solutions were undialyzed and separately prepared from the same batch of venom.

The percentage release of the different mono- and di-nucleotide sequences at various stages of venom and pancreas RNase hydrolysis are shown in Tables XIX and XX respectively, and for this purpose the quantities of the "open-" and "cyclic-ended" mono- and dinucleotides have been combined.

(b) Percent cleavage of PypPy and PypPu linkages after partial RNase hydrolysis.

The end group analyses of products formed by partial digestion with venom and pancreas RNases are shown in Table XXI and they correspond to the oligonucleotide analyses of these same digests summarized earlier in Table XVIII. Ultra-violet contact prints showing the separation of nucleoside diphosphates and residual dinucleotides in PDase digests are shown in Fig. 12. The percentage of PypPu and PypPy linkages cleaved after these three different degrees of partial hydrol-ysis are graphically illustrated in Fig. 13.

A more detailed examination after partial hydrolysis by venom RNase for both 1 and 24 hours is summarized in Table XXII. Data for one hour digests with the pancreas RNase are also included in Table XXII for comparison with the results obtained with venom RNase.

(v) Cleavage of Wheat Embryo sRNA by Venom and Pancreas RNases.

(1) Complete hydrolysis of wheat embryo sRNA by pancreas RNase.

(a) The quantity of PypPu linkages in wheat embryo sRNA.

The end group analysis of fragments produced by complete RNase hydrolysis of sRNA is shown in Table XXIII. The

TABLE XXIII

End Group Analysis of the Products Formed by Complete Hydrolysis of Wheat Embryo sRNA by Pancreas RNase.

Analysis	Nucleosides			Nucleoside Diphosphates		
	A	G	Total	pCp	pUp	Total
	moles/100 moles mononucleotides			moles/100 moles mononucleotides		
1	8.11	6.77	14.9	-	-	-
2	-	-	-	3.28	4.51	7.79
3	2.80	3.38	6.18	4.47	1.71	6.18
	10.9	10.2	21.1	7.75	6.22	14.0

TABLE XXIV

Proportions of PypPu linkages in wheat embryo sRNA.

Linkage	Measured	Calculated
	moles/100 moles mononucleotides	
PypA	10.9	8.3
PypG	10.2	12.3
PypPu	21.1	20.6

TABLE XXV

Mononucleotide and Dinucleotide Analysis of the Products Formed by Complete Hydrolysis of Wheat Embryo sRNA by RNase.

Compound	moles/100 moles mononucleotides
Cp	12.1
Up	4.9
ApCp	2.9
ApUp	1.3
GpCp	2.5
GpUp	1.9

TABLE XXVI

Proportions of PypPy and PypPupPy Sequences in Wheat Embryo sRNA.

Sequence	Measured	Calculated
	moles/100 moles mononucleotides	
PypPy	17.0	17.6
PypApC	2.9	2.2
PypApU	1.3	1.3
PypGpC	2.5	3.2
PypGpU	1.9	1.9

nucleosides measured in analysis 1 were those recovered by PDase hydrolysis of the products of complete pancreas RNase digestion of wheat embryo sRNA. This treatment left an oligonucleotide residue which was eluted together with nucleoside diphosphates, from DEAE-cellulose. Two-dimensional chromatography of this mixture provided for the quantitative measurements of nucleoside diphosphates shown in analysis 2 and for the measurements of dinucleotides contributing to the nucleoside and nucleoside diphosphate values shown in analysis 3. The residue remaining amounted to about 6% of the initial sRNA and, since it contained only oligonucleotides which were larger than dinucleotides, the contribution to end groups could not exceed 2%. The pNp compounds derived from the ends of fragments produced by RNase action on sRNA amounted to about 52% of the purine nucleosides found in analysis 1 and probable reasons for the discrepancy have been mentioned earlier (p.55).

The amounts of PypA, PypG and PypPu linkages, based on the data of Table XXIII, are compared in Table XXIV with the amounts of these linkages calculated for random distribution of mononucleotides in different dinucleotide sequences.

(b) The quantity of PypPy linkages in wheat embryo sRNA.

The mononucleotide and dinucleotide analysis of the products formed by complete pancreas RNase hydrolysis is shown in Table XXV. The components were separated by two-dimensional paper chromatography and the recovery of ultra-violet absorbing material was 95-100%.

TABLE XXVII

End Group Analysis of the Products Formed by Partial Hydrolysis of Wheat Embryo sRNA by Venom and Pancreas RNase.

RNase Concentration per ml Digest	A	G	C	U	Total	pC>p	pCp	pU>p	pUp	Total	%PypPu linkages cleaved
	moles/100 moles mononucleotides										
Venom 0.6 mg	3.41	0.39	0.18	0.27	4.25	1.89	0.53	1.71	0.65	4.78	17
Pancreas 0.02 γ	4.61	1.06	0.55	0.27	6.49	1.71	0.26	2.38	0.64	4.99	26

Activity of venom and pancreas RNase solutions in TCA assay = 29%.

TABLE XXVIII

Quantities of Nucleosides Derived from the -CpCpA Terminus of sRNA after Partial Digestion with Venom RNase.

Nucleoside	mole(s)/100 moles mononucleotides					
	After Venom RNase Hydrolysis		After Alkali Hydrolysis of Oligonucleotides Produced by Venom RNase		Total	After Alkali Hydrolysis of sRNA
A	0.17	+	0.83	=	1.00	1.00
G	0.04	+	0.05	=	0.09	0.02
C	0.01	+	0.27	=	0.28	0.22
U	0.00	+	0.09	=	0.09	0.05
	0.22		1.24		1.46	1.29

The measured proportions of PypPy, PypApC, PypApU, PypGpC and PypGpU sequences are compared with the statistically random proportions in Table XXVI.

(2) Partial hydrolysis of wheat embryo sRNA by venom and pancreas RNases.

(a) Limited hydrolysis of PypPy and PypPu linkages after partial RNase hydrolysis.

The end group analyses of products formed in one hour by partial hydrolysis of wheat embryo sRNA by the venom and pancreas RNases are shown in Table XXVII.

(b) Partial hydrolysis of -CpCpA terminal sequences.

The quantities of nucleosides derived from the -CpCpA terminus of sRNA chains, previously subjected to partial hydrolysis by venom RNase, are shown in Table XXVIII. It would appear from these data that the terminal CpA linkage of sRNA is not rapidly hydrolyzed by the venom endonuclease.

(vi) Hydrolysis of Dinucleotides and Dinucleoside Phosphates by the Venom and Pancreas RNases.

The relative rates at which CpA and UpA linkages in dinucleoside phosphates are hydrolyzed by the venom and pancreas RNases were compared with the rates at which the corresponding linkages are hydrolyzed when they are present in rRNA. For this purpose, the concentrations of the dinucleoside phosphates were adjusted to the concentration of the corresponding sequences in the experiments conducted with rRNA, presuming a statistically random proportion of CpA and UpA linkages in the ribonucleate chains. The effect of a 3'-phosphomonoester substituent on the hydrolysis of dinucleoside phosphates was also examined, and for

TABLE XXIX

The R_A Values of CpAp, CpA, UpA and their RNase Hydrolysis Products.

Compound	R_A Value
CpAp	0.16
CpA	0.29
C>p	0.62
Ap	0.70
UpA	0.74
A	1.00
U>p	1.04

The solvent system employed was 76% ethanol and the development time was 12 hours.

TABLE XXX

Rates of Hydrolysis of CpAp, CpA and UpA by Venom and Pancreas RNase.

[RNase] per ml Digest	RNase Activity in TCA Assay	% Hydrolysis (1 hr)				
		CpAp	CpA	UpA	--CpA--	--UpA--
		0.60 μm	0.66 μm	0.45 μm	0.63 μm	0.46 μm
	%	ml^{-1}	ml^{-1}	ml^{-1}	ml^{-1} (RNA)	ml^{-1} (RNA)
Venom (mg)						
0.13	11	7	-	-		
0.40	25	36	-	-		
0.80	32	-	10	19		
1.2	37	57	-	-	46	62
2.4	46	78	26	40		
4.0	52	83	42	59		
8.0	60	97	64	100		
12.	63	100	78	100		
16.	65	100	81	100		
20.	66	100	84	100	53	102
Pancreas(γ)						
0.0006	0.2	-	-	-	7.5	10
0.006	8	63	-	-		
0.012	16	82	-	-		
0.018	25	85	31	9		
0.030	32	92	60*	21	49	68
0.300	60	100	96	89		

*When [CpA] was increased to 5.9 μm, the percent hydrolysis decreased to 17%.

TABLE XXXI

Rate of Hydrolysis of CpA and UpA by Venom and Pancreas RNase.

RNase Concentration per ml Digest	RNase Activity in TCA Assay	% Hydrolysis	
		CpA	UpA
Venom			
0.8 mg	32%	10	20
4.0	48	42	62
Pancreas			
0.03 γ	32	59	26

[CpA] = [UpA] = 0.7 μm per ml.

this purpose, the dinucleotide CpAp was chromatographically purified from the products of partial alkali hydrolysis of RNA since only the CpA and UpA compounds were commercially available (California Biochemicals). The substrate and hydrolysis products were resolved by direct paper chromatography of digests and the R_A values of the pertinent compounds are tabulated in Table XXIX. The dinucleoside phosphates were hydrolyzed for one hour at only a few selected enzyme concentrations and the data obtained, although incomplete, do provide useful information about the possible design of future experiments. The results of these experiments are summarized in Table XXX, and will be discussed in section (vii). The hydrolysis rates of CpA and UpA for the same substrate concentration are presented in Table XXXI.

(vii) Discussion.

(1) The venom and pancreas RNases.

End group and oligonucleotide analyses of partial hydrolysates of RNA have demonstrated that it is possible to characterize a nuclease activity which is present in only trace quantity in the source material. Thus, although the venom nuclease was only used at a maximum concentration equivalent to about 0.5 γ pancreas RNase per ml of digest, it was nevertheless possible to demonstrate that its action is fundamentally similar to that of the pancreas enzyme. The most notable similarity between the pancreas and venom RNases is the fact that they both cleave only PypPu and PypPy linkages in RNA. Both enzymes cleave PypPu linkages more quickly than the PypPy linkages in RNA but it is apparent that the differences observed are not as great as Witzel and

Barnard noted in their studies of the hydrolysis of dinucleoside phosphates by pancreas RNase. It is notable in this regard that Witzel and Barnard reported maximal hydrolysis rates, whereas, in the present work, the substrate concentration of PypA linkages was limiting from the earliest stages of RNA digestion, except at the lowest enzyme concentration.

It was found that PypA linkages of RNA were hydrolyzed most rapidly by both enzymes but that the venom enzyme cleaved UpA linkages more quickly than CpA linkages whereas the pancreas enzyme hydrolyzed CpA linkages more rapidly than UpA linkages in experiments with dinucleoside phosphates. It was also observed that CpAp was hydrolyzed more rapidly than CpA by both the venom and pancreas RNases and this might indicate that a cationic binding site on the enzymes may account for the more rapid hydrolysis of UpA linkages in RNA chains relative to the rates of hydrolysis observed for the corresponding dinucleoside phosphate.

The preferential scission of PypA linkages accompanying only 1 to 2% cleavage of the total phosphodiester bonds in RNA could be extremely useful for studies designed to fragment rRNA into chains having a mean length comparable to sRNA. The resulting fragments would be relatively homogeneous with respect to chain ends, being mainly $Ap(Np)_nU>p$ and $Ap(Np)_nC>p$, and this homogeneity of terminals would greatly facilitate ultimate deductions about the order in which the fragments occurred in the native macromolecule. The venom enzyme would seem most promising in this respect since there would be a greater proportion of $Ap(Np)_nU>p$ fragments and perhaps by restricting cleavage

more than in the present study or by using different thermal or ionic environments, it would be possible to achieve exclusively $Ap(Np)_nU>p$ fragments with the venom RNase. An additional advantage of the venom RNase over the pancreas RNase in this respect is inherent in the observations that the pancreas enzyme was less restricted to PypA linkages at all stages of hydrolysis since the proportion of PypG cleavages relative to PypA cleavages was higher for the pancreas RNase than for the venom RNase for comparable degrees of endonucleolytic scission of RNA by the enzymes. Partial hydrolysis of sRNA principally at PypA linkages could also prove useful as a means of obtaining fragments large enough to deduce the order of occurrence of smaller fragments obtained by complete hydrolysis. Once again the venom enzyme would seem more promising than the pancreas enzyme for this purpose since it is primarily restricted to PypA linkages even after as much as 4 to 5% cleavage of the total phosphodiester linkages in the RNA.

The general mode of action of both enzymes is characterized by an almost exclusively endonucleolytic attack at PypPu linkages in the earliest stages of hydrolysis and this is followed by a stage in which both PypPu and PypPy linkages are endonucleolytically cleaved. Only after 45 to 50% of the PypPu linkages and 10 to 15% of the PypPy linkages have been endonucleolytically cleaved is there any evidence of an exonucleolytic release of pyrimidine mononucleotides; indeed 70 to 80% of the PypPu linkages and 30 to 35% of the PypPy linkages are broken when only 2 to 3% of the mononucleotides are released by the RNases.

In later stages of hydrolysis, there is a relatively slow exonucleolytic release of Pyp from oligonucleotides composed primarily of contiguous clusters of purine and pyrimidine mononucleotides, i.e. $(Pup)_n(Pyp)_n$. For these later stages of partial hydrolysis, the pancreas enzyme would appear to produce a more useful pattern of oligonucleotides for sequence work since the oligonucleotides found after complete cleavage of PypPu linkages (and about 40 to 50% cleavage of PypPy linkages) would be of the types $(Pup)_n(Pyp)_n$ and $(Pyp)_n$. Comparable digests with venom RNase may contain an additional type of sequence $(Pyp)_n(Pup)_n(Pyp)_n$ since there are still pyrimidine nucleoside residues at unphosphorylated terminals of oligonucleotides (cf. 0.5γ pancreas RNase per ml digest and 20 mg venom RNase per ml digest in Table XXII). Furthermore, it has been noted that some dephosphorylation occurs at very high concentrations of acid-treated venom (20 mg per ml) to yield $(Np)_nN$ fragments and it would thus be desirable to purify the enzyme from this trace of PMase contamination before using it at high concentrations. From the above, it is apparent that of the two RNases, the venom enzyme is more useful at low concentrations and the pancreas enzyme is more useful at high concentrations.

The pancreas enzyme was found to "open" C>p faster than U>p in accordance with the findings of Rushizky *et al.*, but the venom RNase "opened" U>p faster than C>p in the case of both mono- and dinucleotides. This different base dependence for cleavage of 2',3' phosphodiester bonds is in accord with the different base dependence noted earlier for the venom

and pancreas enzymes in cleaving the 3',5' phosphodiester bonds in CpA and UpA. A comparison of the relative rates at which mononucleotides and dinucleotides were released from wheat embryo rRNA by the venom and pancreas RNases revealed that there was a more rapid release of uridine than cytidine nucleotide and that the dinucleotide ApCp (plus ApC>p) was released proportionately more rapidly than other dinucleotides by the pancreas enzyme. The studies of Rushizky et al. (1961) also indicated that the dinucleotide was liberated proportionately more rapidly than any other dinucleotides from yeast rRNA but this was not so in the case of TMV RNA.

The utility of RNases which produce "cyclic-ended" oligonucleotides is limited technically in the case of partial hydrolysates because of the presence of cyclic ends, which complicate fractionation and identification of the hydrolysis products. The recent report (Anraku) of an enzyme which will cleave 2',3' cyclic phosphates, and will not hydrolyze 3',5' phosphodiester bonds, should contribute greatly to the application of partial nuclease hydrolysis techniques in sequence studies.

The TCA assay of whole venom does not provide a measure of the exonucleolytic activity of PDase since the amount of TCA-soluble material is attributable to the action of more than one nucleolytic activity. It is, however, possible to assess the activity of the PDase in whole venom when a specific substrate such as bis-p-nitrophenyl phosphate is utilized. From the known relation between the bis-p-nitrophenyl phosphate and TCA assays for purified PDase preparations, it is then

TABLE XXXII

Proportions of PypPy and PypPupPy Sequences in Rat Liver rRNA.

Sequence	Measured	Calculated
	moles/100 moles mononucleotides	
PypPy	26.9	22.0
PypApC	1.39	2.65
PypApU	0.93	1.59
PypGpC	4.00	4.49
PypGpU	2.26	2.70

Mononucleotide composition of rat liver rRNA: A, 19.3%; G, 32.7%; C, 29.3%; U, 17.6%; Ψ, 1.2%.

TABLE XXXIII

Comparison of Nucleotide Sequences in RNA from Rat Liver and Wheat Embryo.

Sequence		Rat liver rRNA	Wheat Embryo rRNA	Wheat Embryo sRNA
		moles/100 moles mononucleotides		
PypApPy	% total A	12.0	13.4	21.2
PypGpPy	% total G	19.1	17.9	15.0
PypPupC	% total C	18.4	19.8	20.5
PypPupU	% total U	18.1	19.9	20.4
		Ratio of Sequences		
Cp/Up		1.66	1.37	1.67
PypC/PypU		1.96	1.53	2.47
PypApC/PypApU		1.49	1.07	2.23
PypGpC/PypGpU		1.77	1.57	1.31
Gp/Ap		1.69	1.29	1.48
PypGpC/PypApC		2.88	2.06	0.86
PypGpU/PypApU		2.43	1.40	1.46

possible to obtain a measure of the independent exonucleolytic activity of PDase in whole venom from the data in Table VI. Thus, the independent exonucleolytic activity of PDase causes the hydrolysis of 0.25 μm phosphodiester bonds per hour per ml digest at a whole venom concentration of 0.07 mg per ml of rRNA digest. This exonucleolytic activity of PDase is approximately the same as the rate of endonucleolytic cleavage of rRNA by the RNase when it acts independently, since from the data of Table XXII, it is clear that for 0.07 mg acid-treated venom per ml digest, the RNase causes the hydrolysis of 0.17 μm phosphodiester bonds per ml digest during the first hour of its action.

(2) The primary structure of wheat embryo rRNA and sRNA.

The present studies of wheat embryo rRNA can be profitably compared with the results of an extensive series of experiments performed on rat liver rRNA by Lipshitz-Wiesner and Chargaff (1963). Data taken from the paper by the above authors are entered for rat liver rRNA in Table XXXII for comparison with the corresponding analyses of wheat embryo rRNA shown in Table XVI. It is at once apparent that the sequence PypApC is signally low in the rRNA from rat liver just as it was in the case of wheat embryo rRNA. A summary comparison of the sequences in rRNA from rat liver and wheat embryo is presented in Table XXXIII.

From the relative amounts of the sequences PypApPy and PypGpPy, it is apparent that both wheat embryo and rat liver rRNA contain a greater proportion of solitary* G than A.

*Solitary is intended in this case to indicate that the Pu nucleoside is flanked by Py nucleosides in the ribonucleate chains.

A comparison of the relative amounts of PypPupC and PypPupU sequences shows that there is a random probability that a PypPu sequence will be followed by either a C or U residue in the rRNA from both the plant and animal source. The proportion Cp/Up among the RNase hydrolysis products is greater than that found for the constituent C and U nucleotides in the native rRNA from both liver and wheat embryo; furthermore, solitary G in both types of rRNA is followed more frequently by C than U, and solitary A is followed more frequently by U than C when compared with random expectations. Finally, there is a greater tendency for solitary G rather than solitary A to be followed by C and by U than would be predicted for statistical randomness in the rRNA of both sources.

It is regrettable that similar data for rat liver sRNA are not available for comparison with the data for wheat embryo sRNA. There are many obvious differences between the sRNA and rRNA from wheat embryo (Table XXXIII). Most notably, the proportion of solitary A is greater than the proportion of solitary G in sRNA, whereas the rRNA contained more solitary G than A. By contrast with rRNA, it is evident that in wheat embryo sRNA, (i) solitary G is followed more frequently by U than C on a proportional basis, (ii) solitary A is followed more frequently by C than U on a proportional basis, and (iii) there is a greater proportional tendency for solitary A rather than G to be followed by C than would be expected for statistical randomness.

The high degree of correlation between the deviations from randomness that have been noted for the animal and plant rRNA is strongly suggestive of conservative and/or convergent evolutionary mechanisms since it would seem from the data for sRNA that there is no intrinsic enzymic or chemical bias which underlies the specific deviations from randomness observed with rRNA.

Part IV. The Endonucleolytic Cleavage of Wheat Embryo rRNA and sRNA by Snake Venom Phosphodiesterase.

(i) Introduction.

It is apparent from the studies described in Part III that the venom and pancreas RNases display a dominant endonucleolytic action and a relatively weak exonucleolytic action. The fact that endonucleases display exonucleolytic activity raises the question of the possible endonucleolytic action of exonucleases. Several reports in the literature (Felix et al., 1960; Razzell and Khorana, 1959) allude to a slow endonucleolytic attack by snake venom PDase on low molecular weight substrates but there have been no attempts to examine the nature or extent of such cleavages when native rRNA is used as a substrate for the PDase.

It seemed that rRNA would be a particularly interesting substrate to examine since rRNA and sRNA, at the same weight concentration, are hydrolyzed at a similar rate by PDase in spite of the fact that the much greater chain length of rRNA would suggest that the effective substrate concentration (nonphosphorylated chain ends) of rRNA for exonucleolytic attack would be about twenty times smaller than that for sRNA. Factors such as rate-dependence on molecular size and secondary structure could be invoked to account for the similar rates of hydrolysis but it seemed worthwhile to explore the possibility that extensive endonucleolytic scission of rRNA by snake venom PDase might be a contributing

factor. As in the earlier studies where it was found that PDase hydrolyzed thermally fragmented rRNA faster than native rRNA (p.21), it is not possible to directly ascribe the increased rate of PDase hydrolysis to the increased number of chain ends produced by endonucleolytic scission since an alteration of molecular size and secondary structure will also accompany such endonucleolytic cleavages.

The studies by Singer and Fraenkel-Conrat (1963) indicate that the endonucleolytic scission of TMV-RNA by snake venom PDase is dependent on the enzyme/substrate ratio and it is important to note that the present studies were specifically designed to favour endonucleolytic scission so that the frequency and nature of the scissions could be measured by end group analytical techniques.

The native end groups of undegraded rRNA and sRNA can be detected by alkali hydrolysis:

$$(pN)_n = pNpNpN\text{-----}pNpN \xrightarrow[\text{hydrolysis}]{\text{alkali}} 1\,N + 1\,pNp + (n-2)Np$$

where n = 80 (sRNA) and = 1300 (rRNA).

Incomplete exonucleolytic hydrolysis of RNA chains by snake venom PDase would produce no change in the quantity of N and pNp after alkali hydrolysis of these partially digested RNA chains:

$$(pN)_n \xrightarrow[\substack{m \\ \text{exonucleolytic} \\ \text{cleavages}}]{\text{PDase}} (pN)_{n-m} + mpN \xrightarrow[\text{hydrolysis}]{\text{alkali}} mpN + (n-m-2)Np + 1\ N + 1\ pNp$$

If some chains were completely digested by the exonucleolytic action of PDase, then the quantity of N and pNp would be decreased in alkali digests of RNA pretreated by PDase. Indeed, in the limiting situation, where complete digestion of RNA by PDase had occurred, the quantity of N and pNp would be reduced to zero.*

$$(pN)_n \xrightarrow[\substack{n-1 \\ \text{exonucleolytic} \\ \text{cleavages}}]{\text{PDase}} npN \xrightarrow[\text{hydrolysis}]{\text{alkali}} npN$$

Alternatively, if the PDase action on RNA were exclusively endonucleolytic, the quantity of N and pNp would increase directly with the number of chain scissions:

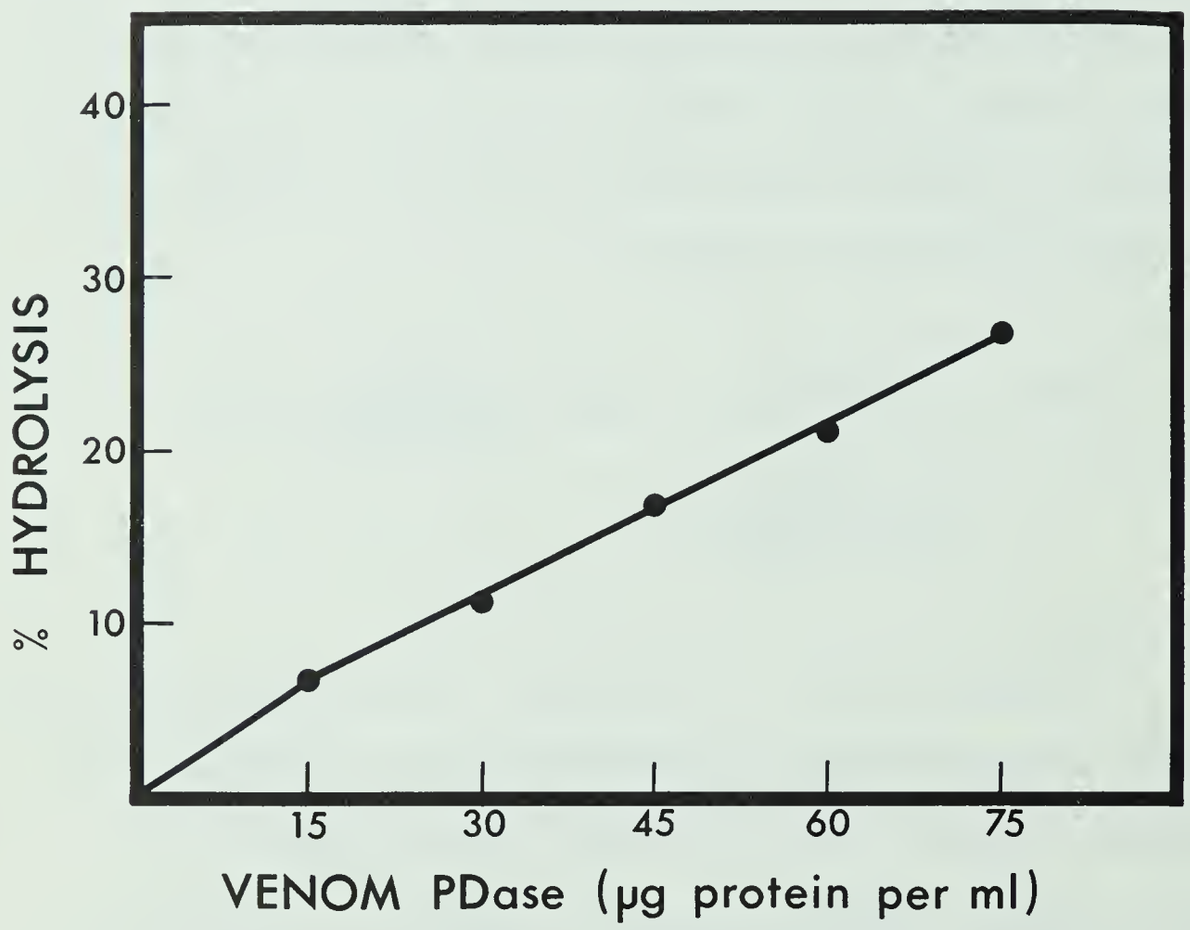
$$(pN)_n = pNpN\text{-----}pNpN\text{-----}pNpN\text{-----}pNpN \xrightarrow[\substack{m \\ \text{endonucleolytic} \\ \text{scissions}}]{\text{PDase}}$$

$$m+1(pN\text{---}pN) \xrightarrow[\text{hydrolysis}]{\text{alkali}} (m+1)N + (m+1)pNp + (n-2m-2)Np$$

---

*This neglects a small amount of N and pNp which would derive from the alkali-catalyzed spontaneous scission of RNA during PDase hydrolysis.

Figure 14



The concentration dependence of snake venom phosphodiesterase activity in the TCA assay

Where both endonucleolytic and exonucleolytic scissions by PDase occur and all RNA chains are only partially digested, alkali hydrolysates will show an increase in N and pNp in direct proportion to the number of endonucleolytic scissions. It should be noted, however, that in practice, measurements of N and pNp are only minimal estimates of endonucleolytic cleavage because, as mentioned above, some chains may become completely digested by snake venom PDase prior to alkali hydrolysis. It is to be expected that the proportion of chains completely digested by PDase will be smaller for less extensive pretreatment with PDase and this expectation is supported by the data which follow.

(ii) Analytical Methods.

(1) Conditions of hydrolysis by snake venom PDase.

RNA and PDase were prepared as described in Part I (ii),(iii) of this thesis. One hundred mg of rRNA or sRNA was dissolved in 13 ml of water and the resulting solution was mixed with 5 ml of 1 M ammonia - 1 M ammonium formate buffer (pH 9.2) and 2 ml of PDase solution (600 γ protein). The digestion mixture was incubated at 37° and at one hour and six hours 10 ml aliquots were removed for analysis. The enzyme concentration dependence of PDase activity in the TCA assay is shown in Fig. 14.

(2) Sedimentation analysis of the hydrolysis products.

A 0.5 ml aliquot of the PDase hydrolysate was precipitated with 1 ml of 95% ethanol, and the resulting precipitate was washed 3 times with 4 ml of 95% ethanol and 2 times

with 4 ml of anhydrous ether. After removing the final trace of ether by aeration, the dry powder was stored at -20° for subsequent sedimentation analysis. The sedimentation analysis was performed at a concentration of 50 μg RNA per ml of 0.1 M sodium chloride-0.01 M sodium acetate (pH 5.5) in a Spinco Model E Analytical Ultracentrifuge equipped with an ultraviolet light optical system. Photographs were taken at 8 minute intervals after reaching a speed of 59,780 rpm and densitometer tracings of the photographs were used to calculate sedimentation coefficients. The sedimentation coefficient so obtained is presumed to be the intrinsic value, $S^{\circ}_{20,w}$ because of the low ribonucleate concentration used for the sedimentation analysis.

(3) The rational and experimental basis for the measurement of exonucleolytic cleavage by PDase.

Each exonucleolytic cleavage of a ribonucleate chain by PDase results in the formation of a 5'-nucleotide. The release of 5'-nucleotides can be measured by paper chromatographic analysis of hydrolysates but since it is found that there is a parallel between the release of 5'-nucleotides and the formation of TCA-soluble ultraviolet absorbing material (260 mμ) during the PDase hydrolysis of DNA (Helleiner, 1955) and RNA, the exonucleolytic action of PDase is more conveniently measured by TCA precipitation of the digests.

A 200 μl aliquot of the PDase hydrolysate was pipetted into 400 μl of a 5% aqueous solution of TCA. The resulting precipitate was removed by centrifugation and the absorbance (260 mμ) of a 1:20 dilution of the supernatant

TABLE XXXIV

The Degree of Hydrolysis, Chain Length and Sedimentation Coefficient of sRNA and rRNA after Incubation for One Hour and Six Hours with Snake Venom PDase.

Substrate	Time of Hydrolysis by PDase (hr)	Percent Hydrolysis of RNA	Total End Groups Detected by Alkali Hydrolysis*			Mean Chain Length of Polynucleotide Fragments	$S^{o}_{20,w}$
			N m%	pNp m%	Ave m%		
rRNA	0	0	0.078	0.082	0.080	1250 (RNA)	18+28
	1	10	0.65	0.91	0.78	115	6.8
	6	43	1.5	1.4	1.4	41	4.1
sRNA	0	0	1.2	1.2	1.2	80 (RNA)	4.2
	1	9	1.4	1.2	1.3	70	4.0
	6	32	2.2	1.9	2.0	34	3.3

*These data have been corrected to quantitative recoveries, the actual recoveries being 70-90% of the values quoted.

solution was measured in order to assess the extent of the exonucleolytic action by PDase.

(4) The rational and experimental basis for the measurement of endonucleolytic cleavage by PDase.

The rational basis for the assessment of endonucleolytic cleavages by PDase has been discussed in the introduction to this part of the thesis. After removal of 0.7 ml for TCA precipitation and sedimentation analysis, the remaining 9.3 ml of the PDase hydrolysate was made approximately 1 M in alkali by the addition of 1.5 ml of 10 M sodium hydroxide, and then incubated for 90 hours at room temperature. The hydrolysate was neutralized with concentrated formic acid and diluted with water to reduce the formate concentration to 0.025 M. The hydrolysate was fractionated on DEAE-cellulose and the isolated N and pNp fractions were desalted and resolved by paper chromatography as described in Part I of this thesis (p.16).

(iii) Results of Analysis.

(1) The extent of exonucleolytic and endonucleolytic cleavage of RNA and the sedimentation properties of the hydrolysis products.

It can be seen from Table XXXIV that after one hour of digestion with PDase there is 10% hydrolysis of rRNA and a nearly 1000% increase in the level of alkali-detectable end groups. The rRNA is 43% hydrolyzed after six hours of incubation with PDase and the level of alkali-detectable end groups is nearly twenty times greater than found before PDase treatment. These data indicate that there have been PDase-

induced endonucleolytic scissions of rRNA chains but do not prove that the endonucleolytic and exonucleolytic activities are necessarily associated with the same protein molecules. Confirmation of the endonucleolytic cleavage of rRNA is forthcoming from the drastic lowering of the sedimentation coefficient that accompanied a relatively small degree of hydrolysis during the first hour of PDase treatment. Thus, there were not more than 14 exonucleolytic cleavages per endonucleolytic cleavage during the first hour of PDase action and not more than 33 exonucleolytic cleavages per endonucleolytic cleavage during the first six hours of PDase action on rRNA. The extent of endonucleolytic cleavage in these experiments is thus much greater than the one endonucleolytic cleavage per 2000 exonucleolytic cleavages observed in studies with low molecular weight substrates.

The end group increment produced by PDase-induced endonucleolytic scission of sRNA is comparable to that observed in the case of rRNA but because of the much smaller initial degree of polymerization in sRNA, relative to rRNA, the decrease in sedimentation coefficient (reflecting molecular size) is much less dramatic in the case of sRNA than it is in the case of rRNA. This difference between sRNA and rRNA is reminiscent of the greater ease with which spontaneous thermal cleavages can be detected in rRNA than in sRNA. For instance, the endonucleolytic cleavage of 0.1 mole per cent of the phosphodiester bonds in each of the two types of RNA corresponds to an average of one break in every chain of rRNA but to an average of only one break in every 10 chains of sRNA.

TABLE XXXV

Relative Amounts of the Different Nucleosides and Nucleoside Diphosphates Produced by Alkali Hydrolysis of rRNA, sRNA and their PDase Digestion Products.

	A	G	C	U	Ψ	pAp	pGp	pCp	pUp
rRNA	21%	31%	24%	24%	0%	9%	42%	23%	26%
PDase 1 hr	23%	23%	26%	15%	13%	21%	26%	17%	36%
PDase 6 hr	21%	24%	29%	14%	12%	22%	33%	16%	29%
sRNA	77%	2%	17%	4%	0%	4%	89%	1%	6%
PDase 1 hr	18%	15%	45%	15%	7%	8%	74%	7%	11%
PDase 6 hr	26%	15%	31%	15%	13%	16%	50%	12%	22%

The nucleoside composition of rRNA (neglecting methyl substituted trace components): A, 24%; G, 31%; C, 25%; U, 18%; Ψ, 1.8%.

The nucleoside composition of sRNA (neglecting methyl substituted trace components): A, 21%; G, 31%; C, 28%; U, 17%; Ψ, 2.8%.

(2) The nature of the endonucleolytic scissions.

If the endonucleolytic chain scissions and the digestion of the resulting RNA fragments by PDase occurred randomly, then the distribution of the bases in the end groups created by these chain scissions should approximate the distribution of bases in the undegraded substrate. The relative amounts of the different nucleosides and nucleoside diphosphates derived from the chain ends of fragments present after partial PDase digestion of sRNA and rRNA are shown in Table XXXV. The most noteworthy feature of these data is the strikingly high proportion of Ψ found among the nucleosides where the proportion of pseudouridine is 7-fold greater than expected for random cleavage of rRNA and 3-5 times greater than expected for random cleavage of sRNA chains.

The preponderance of pUp among the pNp compounds derived from rRNA fragments suggests that there may be some specificity in the endonucleolytic action of PDase but it must be noted that complete digestion of some fragments by PDase could be responsible for the observed bias.

(iv) Discussion.

It is apparent that snake venom PDase exhibits an endonucleolytic activity in addition to its familiar exonucleolytic activity. During the first hour of digestion of rRNA by PDase the rate of exonucleolytic cleavage is not more than 14 times faster than the rate of endonucleolytic cleavage. It is of interest to note that under the conditions of the present study, the endonucleolytic action of PDase was at

least 40 times more rapid than the spontaneous hydrolysis of rRNA at pH 9.2 and 37°.

The preponderance of Ψ at the chain ends of fragments in PDase hydrolysates of rRNA (and sRNA) suggests that: (i) the endonucleolytic scissions by PDase preferentially occur in segments of the RNA chain rich in Ψ, and perhaps at Ψ residues themselves, (ii) that the exonucleolytic action of PDase is arrested at Ψ residues. These possibilities invite further experimentation. It would be interesting, for instance, to determine the sequence of nucleotides adjacent to the Ψ residue since it may be a particular sequence of residues and not Ψ alone that is responsible for the observed endonucleolytic specificity and/or the inhibition of the exonucleolytic hydrolysis catalyzed by PDase. An inhibition of the exonucleolytic action of PDase by pseudouridine, or sequences containing pseudouridine, would have serious consequences for studies of the type conducted by Nihei and Cantoni (1963). These authors presumed that exonucleolytic release of 5'-mononucleotides occurred randomly from the ends of native sRNA chains and concluded from their studies that methylated bases and Ψ were concentrated in the central portion of sRNA chains. It has been shown previously (Part III) that there is a serious danger of contamination with venom RNase in PDase preparations of the type used by Nihei and Cantoni, and the RNase could have introduced endonucleolytic scissions undetected by the methods used by these workers. The endonucleolytic action of PDase itself would have been an additional hazard, not to mention the severe inhibition of PDase hydrolysis by

the N-methyl (Baev et al., 1963) and 2'-O'methyl (Figure 2) substituted components themselves.

The relative amounts of RNase and PDase in whole venom have been shown to be such that the RNase would induce endonucleolytic cleavages at roughly the same rate at which the PDase would induce exonucleolytic cleavages if they were allowed to act independently on rRNA (see Discussion of Part III). Since the PDase has been shown, in this part of the thesis, to induce about 5-10% as many endo- as exo-nucleolytic cleavages in rRNA, the contribution of PDase to the endonucleolysis of RNA by whole venom might be expected to be 5-10% as great as the contribution of the venom RNase itself. Thus, the venom exonuclease (PDase) makes a quantitatively significant contribution to the endonucleolytic capacity of whole venom whereas the venom endonuclease (RNase) makes no significant contribution to the exonucleolytic capacity of whole venom because the exonucleolytic release of pyrimidine mononucleotides by the venom RNase is several orders of magnitude lower than its rate of endonucleolytic action.

It would seem worthwhile to examine the effects of suboptimal pH values, ionic strength and Mg^{++} on the extent of endonucleolytic cleavage by PDase since it might be possible to reduce the proportion of exonucleolytic cleavages and thereby utilize the venom PDase as a primarily endonucleolytic enzyme.

SUMMARY

1. Procedures have been developed for determining the quality and quantity of the nascent chain ends which result from the limited endonucleolytic scission of native rRNA and sRNA chains by (a) a snake venom endonuclease, and (b) a snake venom exonuclease.
2. A venom endonuclease activity, which frequently contaminates partially purified PDase preparations, was recovered free of other nucleolytic activities either by acid treatment or acetone fractionation of Russell viper venom.
3. End group and oligonucleotide analyses of partial RNA hydrolysates have been used as an approach toward the characterization of the endonuclease activity present after acid treatment of Russell viper venom. In spite of the trace amount of the endonuclease present in venom, this approach has demonstrated that the action of the enzyme on RNA is very similar to, but not identical with, the action of the classical pancreas RNase.
4. The pancreas and venom RNases hydrolyze only PypPu and PypPy linkages in RNA, and both enzymes hydrolyze the former linkages more quickly than the latter. Of the PypPu linkages, the PypA bonds in RNA are most rapidly hydrolyzed by both enzymes. The venom RNase has a preferential specificity for UpA linkages in RNA whereas the pancreas enzyme does not manifest a notable preferential specificity between the PypA linkages.

5. The general mode of action of both the venom and pancreas RNases on rRNA is characterized by a rapid initial endonucleolytic attack at PypPu linkages followed, at later stages of hydrolysis, by an endonucleolytic attack at both PypPu and PypPy linkages. After about 40-50% of the PypPu linkages and 10-15% of PypPy linkages have been hydrolyzed, a slow exonucleolytic release of pyrimidine mononucleotides is initiated.
6. The present studies of the pancreas and venom RNases have suggested that limited cleavage of rRNA and sRNA chains by these enzymes will prove useful for sequence work on homogeneous RNA specimens.
7. It has been shown that the familiar snake venom PDase (exonuclease) can display a substantial endonucleolytic action toward rRNA and sRNA, and it was found that there was an unusually high proportion of pseudouridine among the chain ends of the fragments accumulated by partial PDase digestion.
8. Analyses of the mononucleotides and dinucleotides produced by complete pancreas RNase hydrolysis of wheat embryo rRNA and sRNA have shown: (a) striking differences with respect to the random and non-random occurrence of specific sequences in the rRNA and sRNA of wheat embryo, (b) striking similarities with respect to the random and non-random occurrence of specific sequences in the rRNA from a plant (wheat embryo) and an animal (rat liver) source.

BIBLIOGRAPHY

Anraku, Y., J. Biol. Chem. 239: 3412 (1964).

Babkina, G. T., and Vasilenko, S. K., Biokhimiya 29: 268 (1964).

Baev, A. A., Venkstern, T. D., Mirzabekov, A. D., and Tartarskaya, R. I., Biokhimiya 28: 931 (1963).

Bartos, E. M., Rushizky, G. W., and Sober, H. A., Biochemistry 2: 1179 (1963).

Cohn, W. E., and Volkin, E., Arch. Biochem. Biophys. 35: 465 (1952).

Cohn, W. E., Volkin, E., and Khym, J. X., Biochem. Prep. 5: 49 (1957).

Crestfield, A. M., and Allen, F. W., J. Biol. Chem. 219: 103 (1956).

Diemer, J., M.Sc. Thesis, University of Alberta (1965).

Diemer, J., McLennan, B. D., and Lane, B. G., Biochim. Biophys. Acta 114: 191 (1966).

Felix, F., Potter, J. L., and Laskowski, M., J. Biol. Chem. 235: 1150 (1960).

Fraenkel-Conrat, H., and Singer, B., Biochemistry 1: 120 (1962).

Glitz, D. G., and Dekker, C. A., Biochemistry 2: 1185 (1963).

Glitz, D. G., and Dekker, C. A., Biochemistry 3: 1391 (1964).

Glitz, D. G., and Dekker, C. A., Biochemistry 3: 1399 (1964).

Gulland, J. M., and Jackson, E. M., Biochem. J. 32: 590 (1938).

Helleiner, C. W., Ph.D. Thesis, Univ. of Toronto (1955).

Heppel, L. A., Ortiz, P. J., and Ochoa, S., J. Biol. Chem. 229: 679, 695 (1957).

Hurst, R. O., and Becking, G. C., Can. J. Biochem. Physiol. 41: 469 (1963).

Hurst, R. O., and Butler, G. C., J. Biol. Chem. 193: 91 (1951).

Koerner, J. F., and Sinsheimer, R. L., J. Biol. Chem. 228: 1049 (1957).

Lane, B. G., Biochim. Biophys. Acta 72: 110 (1963).

Lane, B. G., Biochemistry 4: 212 (1965).

Lane, B. G., Diemer, J., and Blashko, C. A., Can. J. Biochem. Physiol. 41: 1927 (1963).

Lee, C. Y., and Zbarsky, S. H., Proc. Can. Fed. Biol. Soc. 9: 34 (1966).

Linn, S., and Lehman, I. R., J. Biol. Chem. 240: 1287, 1294 (1965).

Lipshitz-Wiesner, R., and Chargaff, E., Biochim. Biophys. Acta 76: 372 (1963).

Litt, M., and Ingram, V. M., Biochemistry 3: 560 (1964).

Madison, J. T., Everett, G. A., and Kung, H., Science 153: 531 (1966).

Markham, R., and Smith, J. D., Biochem. J. 52: 522 (1952).

McLennan, B. D., and Lane, B. G., Fed. Proc. 24: 602 (1965).

Nihei, T., and Cantoni, G. L., J. Biol. Chem. 238: 3991 (1963).

Ralph, R. K., and Bellamy,A. R., Biochim. Biophys. Acta 87: 9 (1964).

Razzell, W. E., and Khorana, H. G., J. Biol. Chem. 234: 2114 (1959).

Razzell, W. E., and Khorana, H. G., J. Biol. Chem. 234: 2105 (1959a).

Rushizky, G. W., Bartos, E. M., and Sober, H. A., Biochemistry 3: 626 (1964a).

Rushizky, G. W., Greco, A. E., Hartley, Jr., R. W., and Sober, H. A., J. Biol. Chem. 239: 2165 (1964).

Rushizky, G. W., Knight, C. A., Roberts, W. K., Dekker, C. A., Biochim. Biophys. Acta 55: 674 (1962).

Rushizky, G. W., Knight, C. A., and Sober, H. A., J. Biol. Chem. 236: 2732 (1961).

Rushizky, G. W., and Sober, H. A., J. Biol. Chem. 237: 834, 2883 (1962).

Singer, B., and Fraenkel-Conrat, H., Biochim. Biophys. Acta 72: 534 (1963).

Stevens, A., and Hilmoe, R. J., J. Biol. Chem. 235: 3016, 3023 (1960).

Sulkowski, E., Bjork, W., and Laskowski, Sr., M., J. Biol. Chem. 238: 2477 (1963).

Taborsky, G., J. Biol. Chem. 234: 2652 (1959).

Tomlinson, R. V., and Tener, G. M., Biochemistry 2: 697, 703 (1963).

Vanecko, S., and Laskowski, Sr., M., Biochim. Biophys. Acta 61: 547 (1962).

Volkin, E., and Cohn, W. E., J. Biol. Chem. 205: 767 (1953).

Witzel, H., and Barnard, E. A., Biochem. Biophys. Res. Comm. 7: 289, 295 (1962).

Yoneda, M., J. Biochem. (Tokyo) 55: 475 (1964).

Zamir, A., Holley, R. W., and Marquisee, M., J. Biol. Chem. 240: 1267 (1965).